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VITAMIN METHODS

VOLUME I

VITAMIN METHODS

Edited by

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VOLUME I

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FOREWORD

More than five years have elapsed since the publisher proposed the publication of a comprehensive treatise on methods applied to vitamin research, for the purpose of filling a gap in the field. At the outset several principles were laid down, all necessitating painstaking and prolonged work by the coauthors.

In accordance with one of these principles, authors were requested to be as explicit and as simple as possible in their presentations of the various methods. It was thus intended to spare the reader, as far as possible, the necessity of consulting other reference sources before a given assay method could be considered complete and workable. In consequence, not only the scope of the work, but also the length of various chapters increased to such an extent that publication of the material in two consecutive volumes became unavoidable.

The presentation of the material has been built not around the various vitamins, but around the methods employed for assays (physical, chemical, microbiological, and animal assays). This presentation has made it possible to include, without disrupting the organization of the book, chapters on special methods, such as microchemical and clinical procedures, and chapters dealing with the principles and the use of various optical analytical instruments, and with the general technique of animal experimentation. As a result, the material presented in the two volumes of the book covers a wide territory. It may serve not only workers in food-technological, agricultural, chemical, commercial, pharmaceutical, and clinical laboratories, but also physicians interested in problems of nutrition.

Where several alternative methods were available for a given physical, chemical, or biological assay of a vitamin, these have been described in detail, with emphasis, when possible, on the most reliable and suitable procedure. Assay methods, especially chemical, physical, and microbiological methods, often require special modifications, depending on the particular source and form of the vitamin to be analyzed (food products, animal or plant tissues, blood, chemical compounds). These modifications have been incorporated in the various methods.

Compared with the physical, chemical, and even the microbiological methods, animal assays are distinctly more specific in respect to the nutritional value of vitamins. Thus, special emphasis on animal tests appeared to be justified.

With the increasing importance of biometrics as an indispensable tool in experimentation on animals it became essential to give this modern statistical approach a prominent position in the presentation of the material. In the past, the statistical viewpoint received none or only scant consideration in animal assays, even including many of the well-accredited methods. In many instances, revaluation of these methods from a statistical angle has been possible. In other instances existing assays were found to be unsatisfactory. Interested research workers were asked to carry out special investigations which would conform better with modern statistical criteria.

Biometrics is a science in itself, with its own language, not sufficiently well known among workers interested in problems of vitamin assays. The second volume of the book will contain a chapter on statistical methods for biological assays, which does not depend upon prior knowledge of the field and is written so that the inexperienced may make use of modern statistical tools.

The perennial problem of the form in which bibliographical data should be presented has been solved by bringing the references at the end of each chapter in order of their appearance in the text. By this method a number of references pertaining to the same topic were kept in close proximity. Readers who would have preferred the alphabetical order will find a substitute in the Author Index at the end of each volume. The increasing frequency of collaborative papers, with the name of the senior author often hidden in a less prominent position, militates against the usefulness of alphabetical order in bibliography.

Special thanks are due to all collaborators and to the publishers for their patience throughout the greatly prolonged preparation of the book.

PAUL GYÖRGY

October, 1949
Philadelphia

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I. Introduction

Biological assay methods for vitamins are the basis of all research in this field. No progress would have been made at all if such methods had not been developed. However, the animal assay is both time-consuming and expensive to carry out, and it is not surprising that research workers searched for and found more speedy methods for the estimation of vitamins in raw materials and concentrates. These workers naturally turned to some characteristic chemical or physical property of the vitamin on which to base a rapid determination. That they were successful in their search can be seen from the greatly increasing number of publications and research reports that have appeared in recent years. Among the earliest attempts to get away from lengthy animal assays were those in connection with vitamin A. Takahashi *et al.* (1), in 1925, observed a correlation between the ultraviolet absorption spectra of fish oils containing vitamin A and their biological activity. Since then, the use of selective absorption and other physical methods has come more and more to the aid of the research worker in the field of the vitamins.

II. Methods Involving Ultraviolet Absorption Spectra

The term "absorption spectrophotometry" can be defined as the measurement of the intensities of absorption which molecules in the path of light exert at different wavelengths. The spectral absorption curves, which are obtained when the data of such measurements are plotted, depend for their shape on the nature of the absorbing molecules, both of the substance and its environment.

In the study of vitamins, two main regions of the spectrum are involved; (1) the ultraviolet region from 1850-4000 Å and (2) the visible region from 4000-7600 Å. So far the infrared region of the spectrum, from 7600-150,000 Å, has not been used in vitamin assay determinations, but only in structural determinations.

1. NOTATIONS

Lambert's Law states that if I_o is the intensity of light, of wavelength λ, incident upon a homogeneous absorbing medium of thickness l, and I the intensity of the emergent light, then:—

$$log\frac{I_o}{I} = D \text{ (density)} \qquad (1)$$

Beer's Law states that the absorption is proportional to the number of molecules in the light path, *i.e.*,

$$I = I_o.10^{-Ecl.} \qquad (2)$$

Where c is the molar concentration (*e.g.*, of an absorbing solute in a "transparent" solvent), l is the thickness in cm. of the absorbing layer, and E is the molar extinction coefficient.

Eq. (2) can be written

$$Ecl = \log\frac{I_o}{I} = D.$$

$$Ecl = D.$$

$$E = \frac{D}{c \times l}$$

For the full derivation of these equations, the reader is referred to the various texts (*e.g.*, 2, 3, 4). An absorption band is usually described in terms of $\lambda_{max.}$ and $E_{max.}$, *i.e.*, the wavelength in angstroms (Å) or millimicrons (mμ) of maximum absorption, and the molecular extinction coefficient at that wavelength.

The expression E^c_l is the most convenient way of describing an actual measurement. This expression stands for $\log\frac{I_o}{I}$, for a layer l cm. thick of a solution of concentration c. For application to the absorption spectra of natural products, whether mixtures or substances of unknown molecular weight, this expression is usually written $E^{1\%}_{1\,cm.}$ (c being expressed in percentage (wt./vol.) of the solute).

For example, if a layer of $l = 0.5$ cm. of a solution of $c = 0.1$ g./100 ml. transmits 1/10 of the incident light at 325 mμ, then:—

$$D = \log\frac{I_o}{I} = \log\frac{1}{1/10} = 1$$

$$E^{1\%}_{1\,cm.}\ (325\ m\mu) = \frac{D}{c \times l} = \frac{1}{0.1 \times 0.5} = 20.$$

In this form the expression is convenient for the comparison of similar solutions.

In the case of the absorption spectrum of a substance of known molecular weight, it may be convenient to express the absorption in terms of the molecular weight—

$$E_m = \mathrm{E}_{1\,\mathrm{cm.}}^{1\%} \times \frac{M}{10}$$

where E_m is the molecular extinction coefficient and M is the molecular weight.

The data of absorption spectra are usually set down in the form of graphs of the function $E\ (\lambda)$ (E = molecular extinction coefficient and λ = the wavelength). Either E or $\log E$ may be plotted as the ordinate. The wavelengths are plotted as the abscissae, usually in Å or mμ. (1 Å = 10 mμ).

The technique of measuring absorption spectra has been dealt with fully in other places: (2, 3, 4, 5, 6). A method for adapting the Beckman spectrophotometer for use on the micro scale (0.05 ml. of sample solution) has been put forward by Lowry and Bessey (7). See p. 287 ff.

2. VITAMIN A

The existence of vitamin A has been well established for the past 30 years. The importance of this vitamin in health and nutrition has become firmly established during the course of the last quarter of a century, and with this realization, it became more and more necessary to have reliable methods for the accurate determination of this dietary essential. The original biological method is now recognized by the U. S. Pharmacopoeia as the official method for the determination of vitamin A (Vol. II). This method, however, is time consuming and expensive, and a great deal of research has been carried out to find a chemical or physical approach to this problem.

Biological assays on vitamin A preparations take into account only the *biologically available* vitamin A. That is to say, the biological assay determines only that vitamin A or provitamin A which the animal can assimilate. Recent work has shown that, in the case of β-carotene, for example, the animal shows a wide variability in its utilization, depending on the form in which it is administered. This also holds true for other provitamins A.

The most serious defect of the biological assay, beside the fact that it is costly and time consuming, is that it cannot distinguish between vitamin A and the provitamins A.

The chemist, in his determination of vitamin A or provitamins A, aims at the precise determination of the actual amounts present in the product or concentrate. There have been two approaches to this precise analytical determination, (a) the chemical or colorimetric (Carr-Price) method, which will be dealt with in the chapter on Chemical Methods, and (b) physical methods, which depend on the measurement of certain physical constants.

Oser, Melnick and Pader (8) have critically compared the application of the spectrophotometric and the Carr-Price method as applied to fish liver oils. They have pointed out that minor oxidative changes, caused during the preparation of the unsaponifiable fraction, tends to make the colorimetric values low as compared with those obtained from extinction coefficients. These authors point out, however, that it is possible to obtain unsaponifiable fractions without oxidative or mechanical loss.

Any analytical method which is used for the determination of vitamin A, has to cover a wide range of potencies. The following table (1) indicates the range covered by typical vitamin A-containing products.

TABLE I

Source	IU/g.
Milk	about 1
Butter	10-50
Codliver Oil	300-3000
Halibut liver oil	10,000-150,000
Rich concentrates	3,300,000

In vegetable products, the vitamin A activity is due to carotenoid provitamins and, in such products, it is necessary to determine the total provitamin content and the availability of the provitamin to the animal.

The early work on the problem of the spectrophotometric determination of vitamin A is full of anomalous statements and contradictions, and it is only in recent years and particularly since the structure of the vitamin A molecule has been determined that many of these early difficulties have been eradicated.

A. THE VITAMINS A AND THE PROVITAMINS A

Vitamin A, C_{20} $H_{29}OH$, (Fig. 1) is a polyene alcohol derived from the livers of fish, birds and mammals, and from the lining of the intestines of many fish.

In more recent years, further substances possessing vitamin A activity, but distinct from the vitamin, have been discovered. Vitamin A_2 (9) is found in fish liver oils along with A, and vitamin A_3 (10) has been found in mammalian and other liver oils. A fourth substance having certain properties of the A vitamins has been isolated from shark liver oil, the so-called "sub-vitamin A" (11, 12).

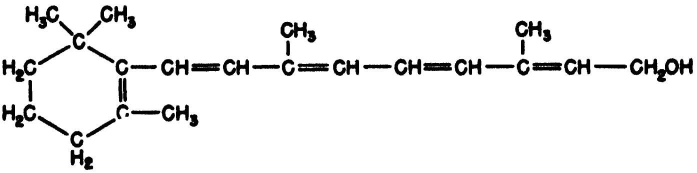

Fig. 1. Vitamin A

The A vitamins all show broad adsorption bands with the following maxima. (Fig. 2, Table II).

TABLE II

	Å	References
Vitamin A	3280	13, 14, 15
Vitamin A_2	3450-3500	9, 16
Vitamin A_3	2850-2900	9, 16, 10
"Sub-Vitamin A"	ca. 2900	11, 12
Neo-Vitamin A	3280	17, 18

Robeson and Baxter (17, 18) have shown that there is a stereoisomer of vitamin A which occurs naturally. This "neo-vitamin A" has substantially the same biological potency as vitamin A. The difference between the two forms of vitamin A is strikingly shown in the appearance of the crystals of the two forms and their esters. The melting points of vitamin A and neo-vitamin A are 63-64° C. and 59-60° C. and the values of $E^{1\%}_{1\,cm.}$ at the maximum absorption in the ultraviolet are 1,750 at 325 mμ and 1,645 at 328 mμ, respectively. These and other properties indicate that the neo-vitamin A is a *cis*-isomer of the better known form. The ratio of the quantity of the neo-vitamin A to that of vitamin A in fish liver oil is about 30: 70.

Shantz, Cawley and Embree (19) have isolated anhydro-vitamin A in crystalline form (m.p. 76-77° C.) by the treatment of vitamin A with hydrochloric acid or antimony trichloride. This compound shows $E^{1\%}_{1\,cm.}$ (371 mμ) 3650, and it has some biological vitamin A activity.

So far, these vitamins A have not been found in any source of vegetable origin. There are, however, certain substances of plant origin which can be converted *in vivo* by the animal into vitamin A. These precursors (provitamins) belong to the class of the polyene pigments, the carotenoids, and all contain one-half of the symmetrical β-carotene molecule- $C_{40}H_{56}$ (Table III).

TABLE III

Carotenoids Having Provitamin A Activity *(Adapted from Morton, (20))*

	m.p.	Double bonds	Optical rotation	CS_2	$CHCl_3$	Petroleum ether	$E^{1\%}_{1\,\mathrm{cm.}}$
α-Carotene $C_{40}H_{56}$	187	11	$[\alpha]^{18^\circ}_{\mathrm{Cd}}$ + 380°	511[a] 478 446	492 460 345, 280	478 447.5 420, 395, 270	1900 ($CHCl_3$)
β-Carotene $C_{40}H_{56}$	184	11	0	520 485 451	493 463 436	485 452 424	2200 ($CHCl_3$) (petrol)
γ-Carotene $C_{40}H_{56}$	178	12	0	533 496 463	510 447 447 in C_6H_6	494, 400 462, 270 432, (348)	*ca.* 2000
Cryptoxanthin $C_{40}H_{56}O$	169	11	0	519 483 452	497 463 433	484 451 425, 405	
Aphanin $C_{40}H_{54}O$	176	11 + 1 CO		533 494	491.5 457 (in CH_3OH)	494 460 432	*ca.* 2000
Myxoxanthin $C_{40}H_{54}O$	168.5	12 + 1 CO		488	one maximum	465	

---

[a] These and other figures in this column denote wavelength of maximum absorption in various solvents.

In recent years, several *cis-trans* isomers of β-carotene have been isolated from natural sources (22) or have been produced artificially (23). Some of these show provitamin A activity, but it is not as yet entirely clear whether this activity is entirely inherent in the isomer or whether it is due to the transformation *in vivo* of at least part of the *cis-trans* isomers into the natural all-*trans* β-carotene.

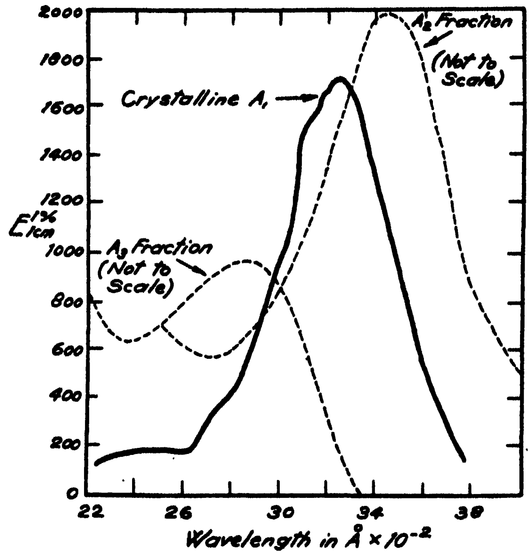

Fig. 2. Absorption spectra of crystalline vitamin A in ethanol, and liver oils rich in A_2 and A_3 (adapted from Loofbourow, (21)).

A further provitamin (Kitol) has been isolated in considerable quantities from whale liver oil (24). It has the approximate formula $C_{40}H_{60}O_2$, and is a dihydric alcohol which has little or no biological activity. On heating, however, to temperatures above 200° C., it yields one mole of vitamin A. Thus, a new type of provitamin A has been added to the previously known group. Kitol shows a single absorption band at 286 mμ and has $E^{1\%}_{1\,cm.}$ 580. It was the first provitamin A to be converted by laboratory means into vitamin A. More recently, Hunter and Williams (25) have converted β-carotene into vitamin A in small yield by chemical means.

B. THE CORRELATION OF ABSORPTION SPECTRA AND BIOLOGICAL ACTIVITY

The first correlation between the growth promoting activity of vitamin A containing oils and the selective absorption in the ultraviolet was noted by Takahashi *et al.* (1). These authors pointed out that such oils exhibited selective absorption in the region of 320 mμ.

The following year Peacock (26) demonstrated that the vitamin A content of an oil was liable to diminish under the influence of light. This observation suggested, to Morton and Heilbron (27), that vitamin A might show selective ultraviolet absorption. These authors carried out a series of quantitative studies in an attempt to correlate the ultraviolet absorption spectra of vitamin A preparations with the color produced by means of a saturated chloroform solution of antimony trichloride. The intensity of this color had previously been shown to vary with the vitamin A content of oils and concentrates by Carr and Price (28).

Morton and Heilbron (27) showed that vitamin A concentrates exhibited a band with a maximum at 328 mμ (325 mμ is now regarded as the maximum for pure vitamin A), the intensity of which showed a good correlation with the color produced in the Carr-Price test, and further that the material responsible for this selective absorption passed into the unsaponifiable fraction of the oil on alkaline saponification.

The view that the absorption band at 328 mμ could be ascribed to the vitamin A in the concentrates was further strengthened when these same authors showed that the extinction coefficient of the 328 mμ band decreased at the same rate as the "chromogenic power" toward antimony trichloride when solutions of vitamin A were irradiated in silvered quartz vessels [see also (29)]. Animal experiments carried out at the same time showed that the decrease in the extinction coefficient corresponded roughly to the decrease in vitamin A content. This correlation between the physical measurements and the biological activity was confirmed on a more quantitative basis by Morton and his colleagues (30, 31, 32).

That the extinction coefficient at 328 mμ was a reliable index of vitamin A potency of an oil was confirmed by Chevallier and Chabre (33), provided the maximum at 328 mμ was not displaced by the presence of absorbing vitamin A decomposition products, in poorly prepared oils.

Final proof that this band was a function of the vitamin A, and not due to an impurity of closely related chemical structure, which accompanied it through the separations, had to await the isolation of pure crystalline vitamin A in 1937 by Holmes and Corbet (13, 34) from the liver oils of *Stereolepsis ishinagi*.

The crystalline vitamin (m.p. 7.5-8.5° C.) showed a single absorption band at 328 mμ with $E^{1\%}_{1\,\text{cm.}}$ 2100 for a freshly diluted solution. However, it was later shown (35) that these crystals were solvated with 1 molecule of methyl alcohol of crystallization and pure vitamin A solvent-free crystals ($C_{20}H_{30}O$) had m.p. 63-64° C. and $E^{1\%}_{1\,\text{cm.}}$ (325 mμ) 1780. (Fig. II)

Baxter and Robeson (35), using a Hilger quartz spectrograph, (Model E-498) with a Spekker ultraviolet photometer and for the light source a tungsten-steel spark, have obtained the following values for the absorption maxima of crystalline vitamin A in various solvents (Table IV).

TABLE IV

Solvent	No. Preparations	mμ [a]	Average $E^{1\%}_{1\,cm.}$
Absolute ethanol	9	326-328	1750 ± 21
Absolute ethanol	1 (3 determinations)	328	1780 [b]
Petroleum (b.p. 30-65°)		328	1760
Ethyl formate		328	1715
Cyclohexane		328	1550
Chloroform		333	1260

[a] The extinction coefficient was measured at the maximum and reported for 328 mμ unless outside the range of 326-328 mμ.

[b] Confirmed by Zscheile and Henry, Purdue Univ. (See also (14)).

Many early attempts to prepare solid crystalline derivatives of vitamin A such as the acetate, benzoate and *p*-nitrobenzoate were unsuccessful (36, 37).

In 1935, however, Hamano (38, 39) obtained the crystalline vitamin A β-naphthoate, m.p. 76° C., and anthraquinone-2-carboxylate, m.p. 124° C. (*cf.* (40)).

Baxter and Robeson (35) have found the following maxima and extinction coefficients for various crystalline derivatives of vitamin A in ethanol (Table V).

TABLE V

Extinction Coefficient of Vitamin A Esters at 328 mμ in Absolute Ethyl Alcohol

Ester	m.p. °C.	$E^{1\%}_{1\,cm.}$ 328 mμ	Equivalent $E^{1\%}_{1\,cm.}$ (328 mμ) Calcd. for vitamin A	$E^{1\%}_{1\,cm.}$ (328 mμ) for vit. A by saponification of esters
Acetate	57-58	1510	1730	1710
Palmitate	27-28	940	1720	1700
Disuccinate	76-77	1240	1420	1700
β-Naphthoate	74-75	1090	1640 [a]	1700

[a] Corrected for the absorption of the naphthoate radical. Ethyl β-naphthoate has $E^{1\%}_{1\,cm.}$ (328 mμ) = 1058.

More recently Ellenberger, Guerrant and Chilcote (40A) have found $E^{1\%}_{1\,cm.} = 1520$ for crystalline vitamin A acetate in isopropanol.

C. THE DETERMINATION OF $E^{1\%}_{1\,cm.}$ (325 mμ)

There are three types of instrument in general use for the spectrophotometric determination of vitamin A—the spectrophotometer, the vitameter, and the photoelectric spectrophotometer. All three types of instrument are capable of reasonable accuracy provided they have been properly calibrated. Gridgeman has stated (41) that, in his experience, "the chance of two observers differing by 5% in the reading of a spectrogram is considerably less than 1 in 100." By using standard conditions, it is possible to determine the E value of vitamin A-containing materials with only a small error. A review of the factors affecting the precision of the spectroscopic determination of Vitamin A has been published by Rawlings and Wait (42).

It is not possible in this chapter to go into the details of the use of the various instruments, and the reader is referred to Brode's (2) and Weissberger's (4) textbooks for such details (see also (43) for details of the Beckman spectrophotometer). (Cf. also page 524.)

Morton and Stubbs (44) have introduced a new approach for arriving at a more precise value for $E^{1\%}_{1\,cm.}$ in the spectrophotometric determination of vitamin A.

The adsorbing characteristics of extraneous materials in vitamin A concentrates modify the shape of the pure vitamin curve. The observed curve is the summation of the curves of the extraneous material and that of the vitamin A.

If measurements of the adsorption are made with a photoelectric spectrophotometer at 3 wavelengths not very far apart, and assuming that the "irrelevant" absorption is linear over this short wavelength range, it is possible to calculate the content of the desired constituent. The calculations may be simplified by choosing as one of the wavelengths the maximum for vitamin A (328 mμ) and the other two are chosen so that their extinctions are equal, *i.e.*, wavelengths on either side of the maximum where the extinction coefficients are $^6\!/_7$ of that at 328 mμ.

Morton and Stubbs (44a) have reported further on this "correction" method.

With a known concentration of oil or concentrate (in a specified solvent), such that $E_{max.}$ for a 1 cm. cell occurs in the region of optional performance for the photoelectric spectrophotometer, E values are determined at the 3 wavelengths, 313, 328, and 338.5 mμ. Normally the values on either side of the maximum are different, and both exceed $^6\!/_7$ of the peak value. The correction is made in two steps as in the example given below:

Cod liver oil, 1% solution in cyclohexane:

$m\mu$	E	$\Delta\ m\mu$	
313	0.640	15	
328	0.712		25.5
338.5	0.620	10.5	

(I) $E_{313m\mu} - E_{338.5\ m\mu} = 0.020$

By similar triangles the correction for slope is:

$$\frac{0.020 \times 10.5}{25.5} = 0.008$$

$$0.712 - 0.008 = 0.704$$

(II) Correction for the height of the general absorption:

$$\frac{0.704 - \chi}{0.620 - \chi} = \frac{7}{6}, \qquad \chi = 0.116$$

$$0.704 - 0.116 = 0.588$$

$E^{1\%}_{1\,\text{cm.}}$ 328 mμ (0.712 gross) = 0.588 (corrected).

From the corrected value at 328 m_μ the whole curve can be constructed by making use of the data contained in Morton and Stubbs' paper (44a). The difference between this curve and the uncorrected curve will represent the spectrum of substances other than Vitamin A, provided that the assumption of linearity over the range $E_{313m\mu}$ to $E_{338m\mu}$ is valid.

The Informal Group which is studying the Physicochemical Determination of Vitamin A by request of the U.S.P. Vitamin Advisory Board, have suggested for collaborative study a method * for Vitamin A determination (44b.). They also suggest making use of the Morton and Stubbs (44a) method of correction. The suggested method is as follows:

Apparatus

Spectrophotometer—An instrument such as the Beckman quartz spectrophotometer or one of equal accuracy and sensitivity shall be employed. It shall be provided with a matched pair of 10 mm. quartz absorption cells.

Reagents

Alcohol. U.S.P. 94.9% by volume.

Isopropanol. U.S.P. XIII, p. 776 Appendix. Redistill if necessary.

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* This suggested method is purely tentative and modifications have since been proposed. The U.S.P. Vitamin Advisory Board are about to approve and publish a recommended method. This method will be included in a supplement of Volume 2 of *Vitamin Methods.*

This reagent must meet the following requirements for spectral purity: When measured in a 1 cm. quartz cell against distilled water it shall show an optical density no greater than 0.01 between 350 mμ and 320 mμ and no greater than 0.05 at 300 mμ.

Sodium Sulfate, Anhydrous. Insoluble matter—Not more than 0.010%. Loss on Ignition—Not more than 0.50%. Neutrality—To pass test. Chloride (Cl)—Not more than 0.003%. Nitrogen Compounds (as N)—Not more than 0.0005%. Arsenic (As)—Not more than 0.0003%. Calcium, Magnesium, and Ammonium. Hydroxide Precipitate—Not more than 0.030%. Heavy Metals—To pass test (limit about 0.0005% lead). Iron—To pass test (limit about 0.001%).

Ether. U.S.P. Must be peroxide free.

Phenolphthalein. Use T.S. U.S.P.

Potassium Hydroxide. U.S.P. Use an aqueous solution 50% w/w.

Saponification

Use non-actinic glassware throughout. Accurately weigh 0.5-1.0 g. oil to be assayed. Boil under reflux, in all glass apparatus (rubber stoppers and corks may not be used) with 30 cc. alcohol and 3 cc. 50% w/w aqueous KOH for 30 min. in a water bath. Cool the solution and add 30 cc. of water. Transfer the resulting solution (which should be clear) to a Squibb-type separatory funnel and extract 4 times with 30 cc. portions of ether. Combine the ether extracts and wash by swirling gently with 50 cc. of water. Repeat washing more vigorously until the last washing gives no color with phenolphthalein (T.S.). Evaporate the ether extract on a water bath to about 50 cc. Add 5-10 g. anhydrous sodium sulfate, stir and allow to settle. Decant into a 100 cc. volumetric flask and make up to mark with ether rinsings of the sodium sulfate. Demonstrate quantitative removal of Vitamin A from sodium sulfate by addition of few drops $SbCl_3$ solution to the residue (dry).

Spectrophotometric Procedure

Evaporate a 25 cc. aliquot of the ether solution of the unsaponifiable extract to about 5 cc. Blow off the remaining ether in a stream of inert gas at room temperature. Take up the residue in sufficient isopropanol to give an expected concentration of 2.7-5.0 γ of Vitamin A/cc. (calculated as vitamin A acetate), or such that will give a density reading in the range 0.4-0.8. Determine optical densities of the isopropanol solution at intervals of 10 mμ in the region 300-350 mμ with a specific reading at 325 mμ.

Calculations

$E_{1\,cm.}^{1\%}$ is the extinction coefficient.
D is the density reading at 325 mμ.
L is length of absorption cell in cm.
C is g. of oil/100 cc. of final dilution.
W is fraction of capsule, tablet, *etc.*, represented in each cc. of final dilution.

$$(1)\quad \frac{E_{1\,cm.}^{1\%}}{0.001540} = \gamma \text{ vitamin A (calculated as acetate)/g. oil.}$$

When sample is a capsule, tablet or other such dosage unit:

$$(2)\quad \frac{D}{L \times 100 \times W \times 0.001540} = \gamma \text{ vitamin A (calculated as acetate)/ tablet, capsule, } etc.$$

Criteria

If the spectrophotometric procedure indicates an extinction ratio E 300 mμ/325 mμ not greater than 0.70, and an extinction ratio E 350 mμ/325 mμ not greater than 0.60 the vitamin A content of sample is that which results from application of formula (1) or (2).

If the extinction ratios are above these values, examine sample by the colorimetric method and calculate vitamin A content of sample by means of formula. If the vitamin A values from the two methods agree within 5%, the spectrophotometric value shall be accepted. If they differ by more than 5%, the lower value shall be accepted.

The standard for vitamin A determinations shall be the U.S.P. Reference Standard for vitamin A (a solution of purified vitamin A acetate crystals in cottonseed oil).

D. CONVERSION FACTORS

It has been shown above that the $E_{1\,cm.}^{1\%}$ of a vitamin A containing material is a linear function of the amount of the vitamin present in the sample. There is, however, some uncertainty about the value of the conversion factor to be used to translate this $E_{1\,cm.}^{1\%}$ into International Units.

In 1931 the first International Standard was set up by the Permanent Commission on Biological Standards of the League of Nations (Health Division). It consisted of pooled crystals of carotene prepared by 7 laboratories. The International Unit was defined as the biological activity of 1 γ of this preparation.

During the next few years, the chemistry of the carotenoids developed rapidly, and it was shown that the standard was a mixture of α-,

β- and probably γ-carotenes. In 1934, the 1931 standard was replaced by a purified sample of β-carotene prepared by Karrer.

In 1934, the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations (45) fixed the unit of vitamin A as the activity of a definite weight, 0.6 γ of a specimen of purified β-carotene. This, dissolved in a vegetable oil at such a concentration that 1 g. contains 0.3 mg. of β-carotene, constitutes the International Standard Preparation of vitamin A. (1 I.U. = 2 mg. of the I.S.P.).

The proper use of the International Standard Preparation involves comparing the biological effect of a known amount with that of a known amount of the substance of unknown vitamin A content.

On the basis of the purified specimens of vitamin A which were available at the time when β-carotene was chosen for the International Standard Preparation, and on further evidence obtained in the examination of fish liver oils, a relationship was established between the absorption coefficient of vitamin A and its biological activity. This factor, known as the "*conversion factor,*" was provisionally set at 1600 (45) and it was made permissible to convert the $E^{1\%}_{1\,\mathrm{cm.}}$ value at 328 mμ to I.U./g. by use of this factor. Thus, an oil showing an $E^{1\%}_{1\,\mathrm{cm.}}$ (328 mμ) of 1.2 would be assayed at $1.2 \times 1600 = 1920$ I.U. of vitamin A/g.

The "conversion factor" of an oil is not normally determinable, but can be estimated by dividing the units/g. obtained in the rat growth bioassay by the $E^{1\%}_{1\,\mathrm{cm.}}$ value at 325 mμ. Such a "conversion factor" is not particularly accurate, since both assays, and particularly the former, are subject to error. Gridgeman (41) has dealt very fully with the subject of the variability of "conversion factors" (see also (20)).

A recent determination of the "conversion factor" has been made by Müller and Reinert (46). The international standard (β-carotene) was compared with vitamin A preparations of widely varying activities as well as crude liver oils. They were first submitted to purification by the methods developed by Müller ((47), see section on chromotographic methods). The result of two independent determinations using very precise conditions yielded "conversion factors" of 1691 and 1712, the mean value being approximately 1700.

Of equal importance to the International Unit, is the U.S.P. (United States Pharmacopoeia) unit. The two are nominally interchangeable, but, the latter being smaller, is commercially accepted.

In the United States, two subsidiary standards have been used. These are samples of cod liver oil. The first, U.S.P. Reference Cod Liver Oil No. 1, was assigned a potency of 3000 I.U./ g. in 1934 by a collaborative assay (48) which calibrated it against the 1931 standard. It has never been officially calibrated against the 1934 International Standard.

The official conversion factor for this reference oil is 2140. However, in 1941 a meeting of 23 commercial and other organizations held in Chicago, adopted a conversion factor of 2000 for trade usage, and this factor was put into effect on June 15, 1941.

A new U.S.P. reference cod liver oil No. 2 superseded oil No. 1 in 1941, which, in contrast to No. 1, bears no official relation (as far as the League of Nations Health Organization is concerned) to the International Standard. Fourteen laboratories (49) using 1665 rats arrived at a potency of 1700 I.U./g. based on the 1934 standard. The E value of the unsaponifiable fraction estimated by 10 laboratories was 0.795. This yields a conversion factor of 2140 (*i.e.,* $\frac{1700}{0.795} = 2140$).

The results of this collaborative assay were not considered sufficiently conclusive to warrant the adoption of a photometric procedure by the United States Pharmacopoeia.

Oser, Melnick and Pader (8) have criticized the subsidiary standard U.S.P. Reference Oil No. 2 on the following grounds.

(1) The ultraviolet absorption curve does not have the characteristic shape of that of vitamin A.

(2) It contains appreciable amounts of irrelevant material which absorbs in the region of 328 mμ.

(3) This absorbing material is not completely removed on saponification.

(4) It is unstable, even when stored under nitrogen and at low temperatures, for a period of one month.

(5) The vitamin A content of the oil and its unsaponifiable fraction, when measured by the spectrophotometric method did not check with the results of the colorimetric method.

In December, 1944, a third reference oil was issued and designated as United States Pharacopoeia reference cod liver oil No. 3.

A recent determination (40A) of the "conversion factor" for 27 fish oils based on this standard has given a logarithmic mean of 2040. This mean value indicates that the U.S.P. Reference Oil #3 has lost potency since its issuance in 1944.

There is a lack of agreement between the British and American "conversion factors," and for a full discussion of this aspect of the vitamin A determination, the reader is referred to Gridgeman (41) and Morton (20).

The extinction coefficient of pure vitamin A($E^{1\%}_{1\,cm.}$ (325 mμ)) has been determined as 1780 (35) which, when multiplied by the usually accepted commercial factor of 2000 gives a value of 3,560,000 units of vitamin A/g. of the pure material. Baxter and Robeson, in conjunction with Harris, have, however, found a value of 4,300,000 units/g. from biological experiments which would require a conversion factor of 2460. There is no valid explanation for this discrepancy and further work is required in order to find a consistent conversion factor.

The U.S.P. has issued the specifications of an oil solution of Vitamin A acetate as the Vitamin A standard and this was officially accepted January 1, 1948. The following is the description of the standard given in the first U.S.P. XIII Sheet Supplement:

Vitamin A Reference Standard.

The cod liver oil which has been used as the U.S.P. reference standard is hereby replaced with an oil solution of vitamin A acetate of the following specifications:

Description:—The standard consists of an encapsulated solution of crystalline vitamin A acetate in cottonseed oil.

The oil solution is assigned a value of 10,000 U.S.P. Vitamin A units/g.

The content of each capsule is assigned a value of 2,500 U.S.P. Vitamin A units.

Reference Oil.—The term "reference oil" in U.S.P. XIII monographs and vitamin A assay is understood to mean the U.S.P. Vitamin A Reference Standard announced in this supplement.

E Value. In collaborative spectrophotometric studies sponsored by the Vitamin Advisory Board it was found that for the whole oil $E^{1\%}_{1\ \mathrm{cm.}} = 5.37$, and for the nonsaponifiable fraction $E^{1\%}_{1\ \mathrm{cm.}} = 5.28$. The solvent used in these studies was dehydrated alcohol, U.S.P. Thus, the conversion factor for calculating biological potency of an oil under assay, based on the E value for the nonsaponifiable fraction and the biological potency of 10,000 U.S.P. Units/g. is 1894. For conversion to a weight basis in terms of mg. of vitamin A/g., the conversion factor of 0.5682 is obtained. The E values for crystalline vitamin A acetate (1545) and for the nonsaponifiable fraction of the oil solution (5.28) have been used to verify the amount of vitamin A contained in each capsule. The oil solution was made to contain 3.00 mg. of vitamin A/g. and by calculation from E values a figure of 2.98 mg./g. is obtained.

Calibrating Spectrophotometric Instruments. The U.S.P. Vitamin A Reference Standard is of value in the calibration of instruments used in the spectrophotometric determination of vitamin A. For this purpose the whole oil is satisfactory. Accurately weigh a portion of the capsule contents, dissolve it in dehydrated alcohol, U.S.P., and make to a final volume such that the spectrophotometric reading falls in the range of

10-40% transmittancy, corresponding to density values of 1-0.4, respectively. Calibrate the instrument by comparing the extinction coefficient obtained with that given above (5.37).

A recent estimation (40A) of the extinction coefficient of the new vitamin A acetate U.S.P. Reference Standard has given the values $E^{1\%}_{1\,\text{cm.}}$ whole oil — 5.24; unsaponifiable fraction — 5.25. The "conversion factor" for 27 representative fish oils was found to be 1500 (logarithmic mean) based on this standard.

E. CALIBRATION AND SPECTROSCOPIC STANDARDS

In the early days of the estimation of the activity of vitamin A concentrates by spectroscopic means, the American Drug Manufacturers Association (50) recommended that the calibration of the density of vitameters and spectrophotometers should be carried out by using the U.S.P. Reference Oil No. 1. By this means, a specific "conversion factor" could be assigned to any instrument, but since it was based on the official 3000 U.S.P. units/g. for this oil and the unwarranted assumption that the $E^{1\%}_{1\,\text{cm.}}$ of the reference oil was a simple function of its vitamin A activity, such conversion factors were incorrect.

It seems obvious that the instruments should be calibrated by means of some spectroscopically pure chemical which has an absorption curve similar in shape to that of vitamin A as well as having a well defined maximum at 325 mμ.

The most widely mentioned substance for use as a calibration standard in the ultraviolet is potassium chromate, but it must have present a dissociation-depressent, such as dilute potassium hydroxide. This substance shows maxima at 272.5 mμ (E 3660) and 371.5 mμ (E 4830). Potassium nitrate is a somewhat better choice, as it shows a maximum at 303 mμ. Its $E^{1\%}_{1\,\text{cm.}}$ is well established at this wavelength and it requires only solution in water.

Neither of these inorganic solutes are satisfactory, since, at 325 mμ, in both cases the absorption curves are sloping steeply.

According to Morton (20), "the perfect calibrating solute will be one which shows a well defined absorption maximum at 325 mμ, is easily accessible in a high state of purity, and is reasonably stable."

Morton suggested two such compounds, (a) *Salicylaldehyde* λ max. (alc.) 325 mμ, log E *ca.* 3.84. A second maximum occurs at 255 mμ, log E 4.0 (b) *Anthraquinone* which possesses an absorption band at 325 mμ in alcohol, and is probably easier to obtain and keep in a state of purity than salicylaldehyde.

Recently, the extinction coefficients of these suggested standards have been determined precisely by Vandenbelt *et al.* (51, 52). These authors

TABLE VI—*Comparison of Extinction Coefficients of Spectroscopic Standards*

Observer	Instrument	Wave-length mμ	Extinction coefficient	Log extinction coefficient
	Potassium nitrate in water			
Von Halban and Ebert (53)	Photoelectric photometer	302	6.98	0.844 [a]
Scheibe (54)	Photoelectric photometer	302	7.0	0.84 [a]
Baly *et al.* (55)	Hilger spectrograph + sector	303	6.92	0.840
	Hilger spectrograph + Judd Lewis photometer	303	6.92	0.840
Ley and Volbert (56)	Spectrophotometer	301	7.063	0.849 [a]
Ewing *et al.* (57)	Bausch and Lomb spectrograph + sector	301	7.00	0.845
Vandenbelt *et al.* (51)	Beckman spectrophotometer	301	7.064	0.849
	Potassium chromate in 0.05 N KOH			
Rossler (58)	Rotating sector + other methods	372	4520	3.655 [a]
		272	3330	3.522 [a]
Morton (59)	Hilger spectrograph + sector	372	4719	3.674
		272	3612	3.558
Hogness *et al.* (60)	Spectrophotometer	371.5	4830 ± 30	3.684
		272.5	3660 ± 40	3.563
U.S.P. collaborative assay, 1940 (61)	Mean, 11 spectrographs (6 types) + photometers	373	4830	3.684
Vandenbelt *et al.* (51)	Beckman spectrophotometer	373	4800	3.681
		273	3688	3.567
	Anthraquinone in ethyl ether			
Morton and Earlam (62)	Hilger spectrograph + sector	325.5 [b]	5624	3.75
Vandenbelt *et al.* (51)	Beckman spectrophotometer	323	4954	3.695
	Salicylaldehyde in ethyl alcohol			
Morton and Stubbs (63)	Hilger spectrograph + sector	325 [b]	3020	3.48
Vandenbelt *et al.* (51)	Beckman spectrophotometer	326	3662	3.564
	Vitamin A ester in ethyl alcohol			
V.O.P.I. collaborative assay (64)	Mean, 17 Beckman spectrophotometers	327	105.6	2.024
	Mean, 11 spectrographs (4 types) + photometers	327	106.1	2.026
Vandenbelt *et al.* (51)	Beckman spectrophotometer	327	106.0	2.025

[a] Estimated from reference curve.

[b] It should be pointed out that the values given by Morton *et al.* for these two compounds are qualitative values.

compared purified preparations of anthraquinone and salicylaldehyde in ethyl alcohol with the normal calibration standards, potassium nitrate in water, potassium chromate in 0.05 *N* KOH, and natural distilled vitamin A ester in ethyl alcohol. The following table (VI) taken from Vandenbelt *et al.*'s article gives their results and compares them with those previously recorded.

The standard compounds were purified in the following manner (51):—

Potassium Nitrate. Reagent quality potassium nitrate was recrystallized twice from redistilled water. The salt was dried at 110° C. with frequent grinding, and stored over Drierite.

Potassium Chromate. The solid was recrystallized twice from 0.005 *N* potassium hydroxide solution. After 1 week air drying at 60° C., with frequent grinding, it was transferred to a suction flask and maintained at 60° C. and 0.2 mm. Hg pressure for a 24-hr. period.

Anthraquinone. The compound was recrystallized 4 times from absolute ethyl alcohol. Melting point 285.6° (corrected).

Salicylaldehyde (*o*-Hydroxybenzaldehyde). The Eastman product was distilled at 0.2 mm. pressure in an atmosphere of nitrogen until consecutive cuts gave a constant index of refraction $n\,{}^{20}_{\mathrm{D}} = 1.5372$.

Vitamin A Concentrate. A 1 ml. ampoule of the natural distilled vitamin A ester, control No. 4673, was obtained from Distillation Products, Inc., in March, 1943, and stored at 5° C. until used.

A third compound, 2-phenylazo-*p*-cresol, has been suggested by Taylor (65). This proposed calibration standard has recently been examined by Kreider (66). The absorption curves for 2-phenylazo-*p*-cresol and vitamin A are practically identical between 290 and 370 mμ. At 328 mμ, $E^{1\%}_{1\,\mathrm{cm.}}$ is 900.

The diazo compound (m.p. 106.5-107° C.) is very stable, and an isopropanol solution did not change its $E^{1\%}_{1\,\mathrm{cm.}}$ after 18 months.

The various proposed standards have been reviewed recently by Rawlings and Wait (42).

F. THE EXTRACTION OF THE UNSAPONIFIABLE FRACTION

The $E^{1\%}_{1\,\mathrm{cm.}}$ of many vitamin A preparations are very low, *e.g.*, many fish liver oils, and such preparations very often contain substances which also absorb in the ultraviolet, and, hence, interfere with the measurement of the $E^{1\%}_{1\,\mathrm{cm.}}$ of the vitamin. To eliminate such interfering impurities, resort must be made to the isolation of the unsaponifiable fractions of such low potency oils. By such a procedure, the interfering substances are, to a great extent, eliminated. The method of preparation of un-

TABLE VII

Extraction of Unsaponifiable Fraction of Fish Liver Oils

(Step-by-step comparison of three methods)

Step	U. S. Pharmacopoeia XII	Kerr-Sorber (Modified)	British Pharmacopoeia (Modified)
Saponification	Reflux 5 g. of oil in 40 ml. of 1.1 *N* alcoholic (95%) KOH for 120 min.	Reflux 5 g. of oil in 35 ml. of 1.8 *N* alcoholic (86%) KOH for 20 min.	Reflux 0.2-1 g. of oil in 15 ml. of 0.5 *N* alcoholic (95%) KOH for 15 min.
Extraction	Evaporate the alcohol. Take up the residue in 50 ml. of water. Extract twice with 50 ml. of ether.	Cool to 30° C. Extract once with 150 ml. of ether.	Add 30 ml. of water. Extract 4-5 times with 30 ml. of ether (Test last extract with $SbCl_3$).
Washing	Once with 20 ml. of 0.1 *N* NaOH. Once with 20 ml. of 0.2 *N* NaOH. Then with 20 ml. portions of water until the last washing gives no color with phenolphthalein.	3 times with 100 ml. of 0.2 *N* KOH. Then with 30 ml. portions of water until the last washing gives no color with phenolphthalein.	Once with 30 ml. of water. Once with 15 ml. of 0.5 *N* KOH. Then with 30 ml. portions of water until the last washing gives no color with phenolphthalein.
Evaporation	To dryness on a steam bath.	To dryness on a steam bath.	To *ca.* 20 ml. on a steam bath. Remove from steam bath, and blow off the remainder of the solvent (do not bring to complete dryness) with a stream of nitrogen.

saponifiable fractions from fish liver oils varies from laboratory to laboratory (20, p. 87), (67, 68).

The three more common analytical procedures for obtaining unsaponifiable fractions, are those of (a) the U. S. Pharmacopoeia (69), (b) a modification of the method of the British Pharmacopoeia (70), and (c) a modification of the Kerr-Sorber method (71). The following table (Table VII), taken from an article by Oser, Melnick and Pader (72), compares the three methods.

The general procedure is as follows:

The sample oil is saponified by means of refluxing with an aqueous alcoholic (86-95%) solution of KOH (0.5-1.8 *N*). The solution is evaporated and taken up in water, or diluted with water, and extracted several times with ether by gentle rotation. The last ether extract should give no color with antimony trichloride by means of a spot-plate test. The ether extract is washed with dilute aqueous KOH solution and then with water until the washings are no longer alkaline to phenolphthalein. The ether is then removed on the steam bath, and the last of the solvent removed in an atmosphere of nitrogen, in order to prevent oxidation.

Using a modification of the British Pharmacopoeia procedure, Oser, Melnick and Pader (72) have shown that the lowering of potency that occurs on saponification of a fish liver oil is not due to incomplete extraction or to oxidation. By resaponification of unsaponifiable fractions, the potency was not further lowered to any appreciable extent.

These authors (72) have investigated the effect of specially purifying the ether and alcohol used in the preparation of unsaponifiable fractions. The general practice has been to use alcohol, free from aldehydes, and peroxide-free ether. They found that, in the case of non-redistilled alcohol (S.D. 30), its use affected neither the extinction coefficient nor the shape of the absorption curve. On the other hand, use of ether (U.S.P. XII) directly from the container, resulted in distortion of the curve and a high extinction coefficient because of absorbing impurities. However, by distilling the ether and discarding the first and last 10% of the distillate, these effects were entirely removed in the case of vitamin A alcohol. These authors also stress the fact that, to prevent oxidative deterioration of the sample, the last few ml. of ether should be removed in an inert atmosphere.

The low results, which are so often obtained in the estimation of the vitamin A content of the unsaponifiable fractions of low-potency fish liver oils, food products and certain pharmaceutical preparations, have been shown to be caused by destruction of the vitamin A by ultraviolet radiations during the preparation of the unsaponifiable fraction from whole oils, which may take several hours (73). The use of colored glassware, which excludes the ultraviolet radiations, obviates this difficulty in the extraction and saponification, *etc.*, of low potency oils (72, 73).

However, other authors have not found this precaution to be necessary (74). It would appear to be good practice for an analyst to determine whether or not precautions against light destruction are necessary under existing conditions in his laboratory.

G. THE EFFECT OF SOLVENTS

The choice of a solvent to dissolve a vitamin A-containing oil for its spectrophotometric determination is important. Solvents affect both the position and the magnitude of the selective absorption bands in a minor but not unimportant way.

It is a well known fact that solutions of carotene in various solvents show differences in the intensity, as well as in the wavelength, of the absorption maxima in the visible, as well as the ultraviolet, region of the spectra. On this basis, it might be expected that vitamin A, a close relative, would show similar phenomena in different solvents. [See also (42)]

The following table (Table VIII), giving data collected from the literature by Gridgeman (41) (see also (75)), shows the variability caused by different solvents, of the intensity and maximum of vitamin A in oils, concentrates, unsaponifiable fractions, and pure crystalline vitamin A.

Of the eight solvents mentioned in the table, two, ethyl alcohol and cyclohexane, received official sanction at the 1934 International Vitamin Conference and in the 1936 "Addendum to the British Pharmacopoeia," for use in the spectrophotometric assay of vitamin A. The use of ethyl alcohol suffers from the disadvantage that vitamin A oils do not readily dissolve in the lower alcohols. Barthen and Leonard (76) have pointed out that isopropyl alcohol suffers from the disadvantage that it has a high coefficient of expansion. Being a lower alcohol, it has also a low solvent power for vitamin A oils. These two solvents suffer from still another disadvantage, in that, being polar, they may give anomalously high E values for unsaponifiable fractions (80).

Gridgeman (41) has indicated his preference for cyclohexane as the most reliable solvent, but he points out that this solvent may give anomalous results since "it has been known to enhance the true E 325 mμ: for example, the E value of the feeding solution of vitamin A β-naphthoate used in the last British coöperative assay was enhanced 11.5% when read in cyclohexane."

Chloroform gives low, and ether high, results when compared to results obtained in alcohol. Smith *et al.* (79) have pointed out that

TABLE VIII

Observed Effects of Various Solvents on $E^{1\%}_{1\,cm.}$ 325 mμ

Reference	Materials	Observation (or inferences) of relative intensities[a]
Hume and Chick (48)	CLO's and unsaps.	EtOH: Cyhxn: $CHCl_3$ = 100: 97: 91
Holmes and Corbet (13)	Vitamin A alcohol crystals	EtOH reading falls on standing
Ewing *et al.* (57)	FLO's	EtOH = *i*-PrOH
Barthen and Leonard (76)	FLO's and unsaps.	EtOH and Cyhxn. give less stable solns. of unsap. than *i*-PrOH
Holmes *et al.* (50)	FLO's	EtOH, Cyhxn. and *i*-PrOH all give "satisfactory results."
Black *et al.* (77)	FLO's	EtOH = *i*-PrOH
Gillam and El Ridi (78)	FLO's and unsaps.	EtOH: $CHCl_3$ = 100: 83 for unsaps., less diff. with whole oils.
Smith *et al.* (79)	FLO's and concentrates.	EtOH: Cyhxn.: Hxn: Ether: $CHCl_3$ = 100: 97.5: 97.5: 107.5: 89
Adamson and Evers (80)	FLO's	EtOH: Cyhxn: *i*-PrOH = 100: 100:89 for unsaps.: equal with whole oils.
Baxter and Robeson (35)	Vitamin A alcohol crystals	EtOH: Cyhxn: $CHCl_3$: P.E.: Et. form. = 100: 89: 72: 100: 98
Morgareidge (67)	FLO's and unsaps.	*i*-PrOH: Cyhxn = 100: 95
Zscheile and Henry (14)	Vitamin A alcohol and ester crystals	EtOH: *i*-PrOH: Cyhxn: Hxn: Ether = 100: 98: 94: 96: 102

[a] Key to abbreviations: EtOH—Ethyl alcohol (usually absolute); Cyhxn—Cyclohexane; $CHCl_3$—Chloroform; *i*-PrOH—Isopropyl alcohol (syn. isopropanol); Hxn—Hexane; Ether—Ethyl ether; P.E.—Petrol ether (syn. light petroleum); Et. form.—Ethyl formate

chloroform causes a shift in the position of the maximum to about 333 mμ, whereas other solvents did not show a detectable shift, except with cyclohexane when the maximum appeared to be nearer 330 mμ than 328 mμ.

Gridgeman (41) suggests that these minor shifts in the maximum of the absorption band of vitamin A are "worth noting because the literature indicates the existence of some confusion between 'maximum' and the much reiterated '328' mμ."

The whole question of solvent effects, and theories regarding these effects, are more fully dealt with in papers by Smith, Stern and Young (79), Adamson and Evers (80), and Zscheile and Henry (14).

Morton attributed the low extinction coefficient in chloroform to the instability of vitamin A in this solvent (quoted in (79)). Smith *et al.* (79), however, on the basis of their experimental results, suggest that the variable results in various solvents is due to the presence of varying proportions of *cis-trans* isomers of vitamin A. Since the polyene structure was established for vitamin A, it has been apparent that variable proportions of geometrical isomerides might exist in different vitamin A-containing materials. So many isomerides are theoretically possible with 5 double bonds that the problem of their occurrence has defied experimental attack, and it has generally been assumed that there is a balancing of effects. Morton (81) has pointed out that, in compounds with one double bond, *e.g.*, *cis* and *trans* stilbene and cinnamic acid (82), the differences in $E_{max.}$ are of the order, 2:1.

There is no complete agreement as to the wavelength at which the extinction coefficient should be measured in the case of fish liver oils. The absorption maxima of pure vitamin A alcohol is now known to be nearer 325 mμ than 328 mμ, but fish liver oils are known to exhibit other maxima in this critical region of the spectrum.

Oser, Melnick and Pader (8) have examined this point in the case of a series of fish oils chosen at random from a large series of samples. Their results are shown in Table IX taken from their paper.

From the preceding table it will be seen that the majority of samples have a maximum between 325 and 328 mμ in isopropanol. The maximum of nonsaponifiable portions of these oils is invariably at 325 mμ. The last column shows that, in assaying the potency of a fish liver oil whose maximum lies between 325 and 328 mμ, the accuracy of the assay is affected no more than 1-2% if the extinction coefficient is determined at the maximum or at 328 mμ.

In the case of atypical oils, usually low in potency, whose maxima lie outside this range (*i.e.*, far from that of vitamin A alcohol) the presence

TABLE IX

Ultraviolet Absorption Maximum of Samples of Fish Liver Oils

(Fish liver oils taken at random from a series of test samples; values are not necessarily representative of each species.)

Sample	$E^{1\%}_{1\,cm.}$ (328 mμ)	Maximum[a] mμ	$E^{1\%}_{1\,cm.}$ (Max.)	Difference[b] $E^{1\%}_{1\,cm.}$ (328 mμ) $E^{1\%}_{1\,cm.}$ (Max.) *Per cent*
Vitamin A acetate	1570	327	1570	0.0
Same, after saponification	1590	325	1620	— 1.9
Distilled vitamin A esters	107.8	328	107.8	0.0
Same, unsaponifiable fraction	106.3	325	107.0	— 0.7
Cod liver oil (No. 1)	0.87	320	0.94	— 7.4
Same, unsaponifiable fraction	0.71	325	0.72	— 1.4
Cod liver oil (No. 2)	1.58	328	1.58	0.0
Cod liver oil (No. 3)	1.14	320	1.27	— 10.2
Same, unsaponifiable fraction	0.98	325	0.99	— 1.0
Dogfish liver oil	6.04	328	6.04	0.0
Shark liver oil	8.02	326	8.11	— 1.1
Soupfin liver oil	56.0	328	56.0	0.0
Black cod liver oil	55.2	328	55.2	0.0
Ling cod liver oil	94.1	328	94.1	0.0
Halibut liver oil	29.5	327	30.0	— 1.6
Tuna liver oil	60.1	327	60.1	0.0
Concentrates (prepared by saponification)	56.7	326	56.9	— 0.4
	96.1	327	97.2	— 1.1
	498	325	508	— 2.0

[a] In isopropanol solution.

[b] Value for $E^{1\%}_{1\,cm.}$ maximum is taken as basis for comparison.

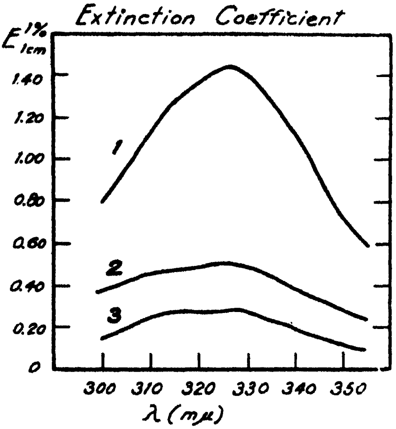

Fig. 3. Curves obtained for (1) vitamin A acetate in ethyl laurate, (2) oxidation of (1) by air, (3) dilution of (1) 1 to 5 with ethyl laurate, by plotting $E^{1\%}_{1\text{ cm.}}$ (after Oser, Melnick and Pader (8)).

of appreciable amounts of irrelevant absorbing material introduces considerable errors.

During the saponification of vitamin A esters, as well as fish liver oils, there is a slight shift in the whole absorption toward the shorter wave-

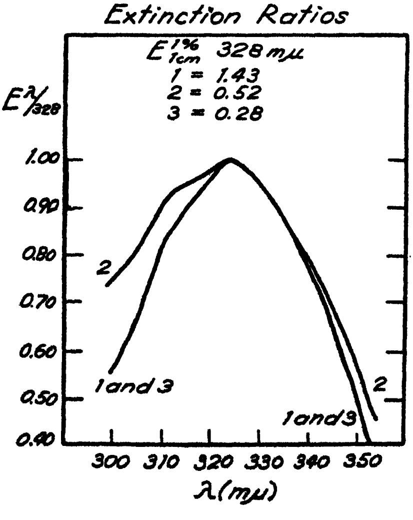

Fig. 4. Curves obtained for (1) vitamin A acetate in ethyl laurate, (2) oxidation of (1) by air, (3) dilution of (1) 1 to 5 with ethyl laurate, by plotting the ratio E/328 (after Oser, Melnick and Pader, (8)).

lengths. In the case of pure vitamin A esters, this shift in the absorption maximum is from 328 mμ to 325 mμ. To simplify the comparison of curves of whole oils and esters with their unsaponifiable fractions, Oser, Melnick and Pader (8) have suggested that, in plotting the curves of vitamin A absorption spectra, the ordinate represents the extinction ratios (E λ/328) rather than the extinction coefficient against the wavelength (λ). By the extinction ratio is meant the ratio of the extinction coefficient or photometric density at a given wavelength to that at 328 mμ, the generally accepted absorption maximum for naturally occurring vitamin A esters. By this means, it is possible to obtain curves, regardless of vitamin A content, which lend themselves to direct comparison with that of a pure vitamin A preparation.

This technique is illustrated in Figs. 3 and 4 above.

H. CHROMATOGRAPHIC PURIFICATION

Purification of vitamin A preparations has been accomplished by chromatographic means. The use of such methods has made possible the spectrophotometric determination of vitamin A in the presence of large quantities of light-absorbing impurities such as the carotenoids, *etc.*

Wilkie and de Witt (83), in the determination of vitamin A in margarine, have purified their extracts by chromatographing them on a column of celite and magnesium oxide. Layers of hydrosulfite were placed at the top and bottom of the column to guard against oxidation. The passage of the vitamin through the column was followed by making use of the green fluorescence shown by vitamin A in ultraviolet light (84, 85, 86, 87).

The separation of vitamin A from carotenoids can be accomplished by making use of the fact that the latter, in petroleum ether solution, are less strongly absorbed than vitamin A on activated alumina, and are washed through the column, leaving the colorless vitamin on the adsorbent (88, 89).

Vitamin A has also been purified by absorption on magnesia after preliminary absorption on activated charcoal (90, 91), Fuller's earth (92), calcium carbonate (93), and sodium carbonate (94, 95).

Mann (96) has described a simple method for the separation of vitamin A from α- and β-carotene.

The unsaponifiable fraction is dissolved in petroleum ether (b.p. 40-60° C.) and poured into a small column of defatted bone meal. Carotene (α- and β-) readily washes off the column with petroleum ether and the eluate becomes colorless. Vitamin A, carotenol, and artefacts, are rapidly eluted by small washings with acetone, and can be further separated by partition methods.

The technique of chromatographic analysis has been adequately described elsewhere, and the reader is referred to the texts of Strain (97) and Zechmeister (98).

Vitamin A may be separated from its esters by chromatographic means. The vitamin is much more readily adsorbed on activated alumina than its esters (47, 99, 100).

An analytical method for the separation of the vitamin alcohol from its esters has been devised by Reed, Wise and Frundt (101). The mixture of vitamin alcohol and esters, in ethylene dichloride solution, is chromatographed on a column of activated alumina. The chromatogram is developed with further quantities of ethylene dichloride. By this means, the esters are eluted, leaving the free vitamin A alcohol adsorbed on the column. The original solution and the eluate are assayed by appropriate means and the percentage of free and esterified vitamin in the original oil calculated from these results.

The use of the chromatographic purification step is illustrated in the determination of vitamin A in oleomargarine devised by Wilkie and de Witt (83). In this assay, the unsaponifiable fraction of the margarine is chromatographically analyzed from petroleum ether on a mixture of celite and magnesium oxide. The chromatogram is developed and the vitamin A band is followed by its fluorescence in weak ultraviolet light.

Since oleomargarine is variable in composition, Wilkie and de Witt make use of a recovery correction.

The method in detail is as follows:

(a) *Saponification and Extraction.* Weigh out 10 g. of oleomargarine into a tall 300 ml. beaker and add 50 ml. of boiling 95% ethanol. Stir until the sample is completely disintegrated, and add 0.25 g. of anhydrous Na_2SO_3. Cool the solution to 35° C., and add 25 ml. of 50% aqueous KOH solution. Stir continuously for 5 min. and allow to stand at room temperature for 15 min. (stirring occasionally).

Transfer the solution to a 500 ml. separatory funnel. Rinse the beaker with 50 ml. of a freshly prepared 5% solution of $Na_2S_2O_4$ and add the rinsings to the saponification mixture. Add 100 ml. of petroleum ether,[1] shake vigorously, and allow to stand for 5 min. Drain the aqueous portion into another separatory funnel, and extract with 50 ml. of a mixture of 90% petroleum ether and 10% U.S.P. ethyl ether. Allow to stand for 3 min. Transfer the aqueous layer to another separatory funnel, and extract as before. Make a total of 7 such extractions and combine the ethereal extracts in the separatory funnel containing the first extract. If a 3-layer system develops at any point, continue the extractions with 50 ml. portions of petroleum ether alone. In such 3-layered systems, transfer only the top layer to the original petroleum ether extract.

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[1] The petroleum ether should be tested by measuring the transmission in a spectrophotometer at 300 mμ. A 1 cm. quartz cell should be used. No cell is used as a blank in this test. An ether having a transmission greater than 85% can be regarded as satisfactory, although the best grades should give a reading of 90%.

Pour through the combined ether extracts two 200-ml. portions of distilled water, and separate off each portion, without shaking. Make 5 additional washings with 20 ml. portions of 5% aqueous $Na_2S_2O_4$, shaking vigorously and allowing the solution to stand for 2 min. before the removal of the water layer. To the combined ether extracts add 200 ml. of distilled water (do not shake or stir), and drain off the aqueous layer. Repeat this washing with another 200 ml. portion of distilled water. Allow to stand 10 min., then drain off the aqueous layer. Filter the ether extract rapidly through 25 ml. of a powdered mixture of anhydrous Na_2SO_4 (90%) and Na_2SO_3 (10%). Evaporate the filtrate on a steam bath to less than 10 ml., transfer to a 10 ml. volumetric flask, and make to volume with petroleum ether.

(b) *Chromatography.* Prepare the adsorption column by using an Allihn sugar tube, or similar apparatus. It consists of a fritted filter of medium porosity fitted in a tube about 20 mm. in diameter and about 10 cm. long. The lower end of the tube is constricted to permit ready attachment to a 250 ml. suction flask by means of a rubber stopper. Place a 3 mm. layer of $Na_2S_2O_4$ directly upon the fritted disc, apply suction, and add the adsorbing material. (A 3 : 1 mixture of purified diatomaceous earth (Celite) and MgO (Baker's analyzed) is a suitable adsorbent.) Tamp the adsorbent lightly with a flared glass rod as it is added in small quantities until it has an approximate depth of 2.5 cm. Then add a 5 mm. layer of $Na_2S_2O_4$, followed by a 1 cm. layer of Na_2SO_3.

After wetting the column with 30 ml. of petroleum ether, add rapidly a 5 ml. aliquot of the extracted sample. When the solution is about to disappear into the surface of the column, add more petroleum ether until the vitamin A appears as a fluorescent band visible under weak ultraviolet light.[2] Elute this band with petroleum ether alone, or with a 0.2% solution of glacial acetic acid in petroleum ether, until it is approximately 5 mm. from the end of the absorbing column. Discard the eluate that has passed through, connect the end of the column to a clean 250 ml. suction flask, and complete the elution. (The elution may be regarded as complete when the vitamin A fluorescence is no longer evident.) Transfer the eluate to a 50 ml. beaker, evaporate to less than 10 ml., transfer to a 10 ml. volumetric flask, and adjust to volume with petroleum ether.

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[2] A convenient source of ultraviolet light may be prepared by mounting a 2.5 watt General Electric argon bulb in a metal housing fitted with a suitable light filter. The bulb should have a double contact bayonet base and no resistor in the base. A 500 ohm resistor is connected in series with the lamp for operation on the 110 volt AC lines. A switch should be provided for continuous operation, and a push button for intermittent operation. No brighter source of ultraviolet should be used. The bulb is mounted on an adjustable clamp so that it can be brought as close as possible to the exit filter, which is a polished 2 in. square of No. 986 glass as provided by Corning Glass Works.

Determine the optical density of this solution at 5 mμ intervals over the range from 270 to 450 mμ, and calculate the vitamin A potency of the oleomargarine by the formula:

$E^{1\%}_{1\,\text{cm.}}$ (340 mμ) × 2500 = U.S.P. units of vitamin A/g. of margarine.

Since oleomargarine is variable in composition, and all of the limitations of the method are not known, it may be desirable in some cases to conduct recovery experiments in order to determine the possible loss of vitamin A during the procedure. This may be done by taking duplicate samples. To one sample add 1 ml. of a petroleum ether solution of vitamin A (150-200 units/ml.) just before the addition of KOH during saponification. (This petroleum ether solution was found to be stable at room temperature over a period of several months, when kept in an amber bottle over Na_2SO_3.) Then assay both samples according to the above procedure, and calculate the per cent recovery of added A as follows: *Recovery Calculation* — $2[(D340 \text{ standard} + \text{unknown}) - (D340 \text{ unknown})] = D'340$, the density of the standard after processing, that is to say, equivalent to 1 ml. of standard $Ds340$ made to 10 ml.

$$\text{Recovery } \% = \frac{D'340}{Ds340} \times 100$$

The use of Floridin XXS as an absorbent in the determination of vitamin A in margarine and vegetable oils has been advocated by Awapara *et al.* (102). Vitamin A is the only substance, absorbing between 300-350 mμ, which is adsorbed on Floridin.

The following method has been applied to margarines and vegetable oils. A 5 g. sample is saponified for 30 min. with 5 ml. of 50% KOH and 20 ml. of 95% ethyl alcohol. The hydrolyzate is made to a total volume of 80 ml. with water and alcohol to give a final alcohol concentration of 50%. Extraction is carried out for 3.5 hr. with 120 ml. of Skellysolve A in a continuous extractor. The petroleum ether extract is washed twice with water, three times with 0.5% sodium hydrosulfite, and four times more with water. The petroleum ether is then removed by evaporation in a stream of nitrogen, and the residue taken up in 10 ml. of benzene. Five ml. of this solution are diluted to 25 ml. with benzene, and the spectrophotometric measurements are made at 300, 332, and 350 mμ. The remaining 5 ml. of benzene solution are poured onto a layer of purified Floridin, 4 mm. thick, in a chromatographic column, 20 mm. in diameter. The Floridin is first wet with 5 ml. of benzene and the extract added carefully before the benzene has disappeared from the column. (When considerably thicker layers of Floridin were used, it was found that substances other than vitamin A were removed from the extract and the results were inaccurate.) The column is then washed with about 15 ml. of benzene, the combined washings are made up to a volume of 25 ml., and readings made on the spectrophotometer at the wavelengths indicated above. The calculation of vitamin A concentration is made from the difference between the readings for the two solutions at 332 mμ, and the differences between the readings at 300-350 mμ serve to demonstrate the completeness of removal of the interfering substances. In calculating the vitamin A, it must be remembered that its absorption curve in benzene differs from the curves in petroleum ether or alcohol and the relation

$$\text{Vitamin A in I.U./g.} = \frac{(\text{extinction difference at } 332\ \text{m}\mu)\ (50).}{0.0442\ (\text{weight of sample})}$$

has been used.

The extinction difference is presumed to represent the extinction due to the vitamin A removed by the Floridin; the factor 50 corresponds to the final total volume in which the nonsaponifiable fraction is dissolved; the factor 0.0442 is the value taken for the extinction of a solution containing 1.0 I.U. ml. This factor is based on the usual conversion figure of 2,000, corrected for the difference between absorption in benzene at 332 mμ and petroleum ether at 328 mμ.

I. THE QUANTITATIVE SEPARATION OF VITAMIN A ALCOHOL AND VITAMIN A ESTERS

Müller (103, 104) has developed methods for the determination of the activity of an adsorbent by measuring the heat produced when the adsorbent is mixed with the solvent. Making use of these standardized absorbents, this author (47) has published an improved technique for the chromatographic purification of vitamin A which yields preparations free from interfering substances. Müller makes use of chromatographic columns packed with three layers of Al_2O_3, having different activities; the lowest layer being the most active adsorbent. The two upper layers vary in activity depending on the particular problem, *i.e.*, the separation of vitamin alcohol, or vitamin A ester, or β-carotene. Vitamin A alcohol and ester are easily separated by this technique, but to effect a separation of the ester and β-carotene, the ester must first be saponified. Mixtures of these three are completely separated by combined saponification and chromatography. The limits of the method are 40 I.U. of vitamin A and 10 of β-carotene/g. of assay material. For a preparation containing

1000 I.U./g. of vitamin A, the limit of error is ± 10%; containing 500 I.U./g. or less, ± 20%; for β-carotene, ± 5%.

Reti (105) has shown that substantially all the vitamin A in fish liver oils was present in an esterified form, by means of distribution of the oils and their unsaponifiable fractions between immiscible solvents.

The analytical separation of vitamin A from its esters in fish liver oils has been carried out by means of molecular distillation (106).

Gillam (107) reported the distribution of vitamin A alcohol between aqueous methanol and petroleum ether. Claussen (108) separated the alcohol from its esters by distribution between hexane and 81.9% ethanol.

A quantitative determination of the esterified vitamin A in fish liver oils has been described by Reed, Wise and Frundt (101), who used distribution between 95% aqueous methanol and petroleum ether. These authors found that 27% of the free vitamin and 98% of vitamin palmitate remained in the petroleum ether phase.

A modified procedure has been described by Kascher and Baxter (109), who use distribution between 83% aqueous ethanol and petroleum ether for the estimation of the esterified vitamin A in fish liver oils. By this means, they found that of the five extractions of crystalline vitamin A alcohol, 97% was found in the combined alcoholic extracts. In a similar experiment using a refined cottonseed oil solution of crystalline vitamin A palmitate, 3% of the ester passed into the alcohol extract.

The procedure described by these authors is as follows:

A weighed sample of fish liver oil or concentrate containing a total of about 20,000 units of vitamin A is dissolved in 20 ml. of petroleum ether (Skellysolve F) in an amber glass separatory funnel (125 ml.). This solution is extracted five times with 20 ml. portions of 83% ethanol (83 ml. of absolute ethanol plus 17 ml. of water) which has been previously saturated with petroleum ether. The two phases are shaken together for at least one minute in each extraction.

The five ethanol extracts are combined, and the volume is adjusted to 100 ml. A suitable aliquot (2 ml. if a high ester content is expected in the sample) is evaporated at 40° C. in a current of nitrogen in an Evelyn colorimeter tube and the residue dissolved in 1 ml. of chloroform for assay by the antimony trichloride reagent (110).

The petroleum ether layer is adjusted to a volume of 100 ml., and a further dilution (usually 1:10) is made. One milliliter of this final dilution is evaporated under nitrogen in an Evelyn colorimeter tube. The residue in 1 ml. of chloroform is assayed by the blue color method.

This method was standardized by a distilled concentrate, whose biological potency (units/g.) had been calculated by multiplying the extinction coefficient ($E^{1\%}_{1\text{ cm.}}$ 328 mμ) by the conversion factor of 2000. A calibration curve was constructed relating galvanometer readings to units in the aliquot tested.

Since 3% of the vitamin A alcohol remains in the petroleum ether layer and 3% of the esters are extracted by the 83% ethanol, the number of units of esters, E, and of alcohol, A, in the sample can be calculated by the formulas:

$$E = 1.032y - 0.032x$$
$$A = 1.032x - 0.032y$$

where x = total units in ethanol phase
y = total units in petroleum ether phase

The percentage of the vitamin A esterified in the sample is calculated by the formula:

$$\% \text{ vitamin A esters} = \frac{100E}{A+E}$$

The presence of esters of low molecular weight, such as the acetate, would lead to error in using these formulas, since their distribution properties are different. Molecular distillation of many fish liver oils, however, has indicated that only the higher fatty acid esters of vitamin A are present.

By this means these authors have shown, in a series of 12 fish liver oils and three distilled concentrates of varying potency, that 96-100% of the vitamin A is present in the esterified form.

It should be pointed out that impurities may affect such distributions, *e.g.*, Gillam has shown that 1% of cholesterol affects the distribution considerably.

J. METHODS OF SPECTROSCOPIC VITAMIN A ASSAY

Two general methods for the determination of vitamin A by means of the spectrophotometer have been used. The first is the *"direct spectrophotometric"* determination, which is somewhat limited in its usefulness, and is usually used for high potency oils and for the unsaponifiable fractions of low potency oils, or for vitamin A preparations where the interfering absorbing impurities can be estimated separately. For example, in the determination of vitamin A in foodstuffs, *etc.*, the unfortified foodstuff can be used as a control. By subtracting the $E^{1\%}_{1\,\text{cm.}}$ determined for the extract of the latter from that obtained for the extract of the fortified sample, the determination of the $E^{1\%}_{1\,\text{cm.}}$ of the added vitamin A can be arrived at.

The second general method is the so-called *"destructive irradiation technique,"* whereby a blank sample is obtained by the destruction of the vitamin A contained in part of the sample by irradiation with ultraviolet light. This method has been used for determining the vitamin A content of food products and animal tissues, since the direct spectrophotometric method is useless because of the large amounts of non-vitamin A absorbing material present in such samples.

(1) *The Direct Spectrophotometric Method*

The procedure for the direct spectrophotometric determination of vitamin A consists of dissolving fish liver oils (or other oils) in a suitable solvent and determining quantitatively the amount of ultra-violet light absorbed by the sample under test.

A portion of the oil to be assayed is accurately weighed into an amber-colored volumetric flask. The oil is introduced by means of a volumetric pipet. Then 15-20 ml. redistilled isopropyl alcohol is added until the oil is dissolved. The solution is then made to volume with isopropyl alcohol, and the flask thoroughly agitated to insure uniform solution. Dilutions to suitable concentrations may be made from this solution. The assay should be made within 5-10 m. after solution is complete.

Using the Bausch and Lomb medium quartz spectrograph with a Bausch and Lomb rotating sector photometer, the absorption cell is filled and placed in the spectrophotometer. The solution cell is placed in the non-variable sector beam, the solvent cell in the variable sector beam. A series of exposures are made with known successive reductions of the percentage of light passing through the solvent cell. The photographic plate is processed, dried and examined for the position at which the absorbed and the reduced spectra are of equal blackness or density. The extinction coefficient of the absorptive maximum is then calculated from the formula:

$$E^{1\%}_{1\,\text{cm.}} = \frac{\text{Log}\,\frac{I_0}{I}}{l \times \%\ \text{concentration}}$$

where $E^{1\%}_{1\,\text{cm.}}$ is the specific extinction coefficient of a 1% solution in a 1 cm. cell. I_0 is the incident light intensity, and I the transmitted light; l is the length of light path through the absorption cell in cm.; per cent concentration is the weight of the sample times 100 divided by the milliliters of solution. If the Beckman spectrophotometer is used, the assay solution is placed in one cell and the second cell is filled with the same solvent as used to make up the assay sample. Density readings are then determined from 300 to 350 mμ, and the extinction coefficient of the absorption maxima is then calculated from the formula:

$$E^{1\%}_{1\,\text{cm.}} = \frac{d}{l \times \%\ \text{conc.}}$$

where d is the density, l the thickness of the cell in centimeters and % concentration is the weight of the sample in grams in 100 cc. of solvent.

In the case of oils containing less than 10,000 U.S.P. units of vitamin A/g., the unsaponifiable fraction is obtained by the following method:

One g of the oil is accurately weighed and saponified with 3 ml. of 50% KOH and 30 ml. of 95% ethyl alcohol by refluxing on a water bath until the solution is clear. This saponification usually takes from 7 to 15 min. The solution is then cooled and 30 ml. of distilled water added, part being poured down the reflux condenser. The solution is then transferred to a separatory funnel and extracted with three quantities of 25 ml. of ethyl ether. The combined ethereal extracts are washed, first with two 20 ml. portions of distilled water, then with two 20 ml. portions of $N/2$ KOH, and again with two 20 ml. portions of water. These washings should be carried out by means of gentle rotation of the separatory funnel rather than by shaking, as this limits emulsification. The washing should not be separated until all the emulsion at the interface is broken. In the case of a resistant emulsion, it may be necessary to add more ether to obtain a sharp separation. The washing and shaking is continued until no turbidity is produced in the washings when acidified with 10% HCl. The ether layer is then allowed to stand for 10 min. and any water that separates is then discarded. The ether solution is evaporated on the steam bath almost to dryness, the last few ml. being removed in an inert atmosphere. The residue is taken up in isopropyl alcohol (or other suitable solvent) and then made up to suitable dilution for the determination of $E^{1\%}_{1\,\text{cm.}}$.

Because of the extraneous pigments present in dehydrated eggs, a suitable correction must be made in the spectrophotometric assay of vitamin A when applied to such products.

Previous investigators (111, 112, 113) have found that the pigments in eggs, from hens on a normal diet, contain over 90% of a mixture of luteol and zeaxanthol and small amounts of cryptoxanthol and carotene. Schrenk, Chapin and Conrad (114) have investigated the pigments of dehydrated eggs by chromatographic and spectrographic analysis, and found that luteol accounted for 63-70% of the total pigments in different samples: zeaxanthol 32-20%, cryptoxanthol 3-5%, and carotene 2-4% of the total.

Basing their calculations on the fact that 90% of the pigments were luteol plus zeaxanthol, these authors obtained a correction factor to be deducted from the total absorption at 326 mμ, of 15%.

Extraction of Vitamin A. A 10 g. sample of dehydrated egg is placed in a Waring Blendor and extracted for 10 min. with 100 ml. of peroxide-free ether. The sample is filtered on a Buchner funnel and washed with several small portions of ether. The egg powder is then replaced in the Blendor and re-extracted with 60 ml. more of ether for 2 min. A third extraction for another 2 min. is usually required. This extraction procedure removes all the vitamin A and practically all the yellow pigments.

Preparation of Extract for Analysis. The combined ether extracts are placed in a 500-ml. round-bottomed flask, to which a few glass beads have been added to prevent bumping, and evaporated on a steam cone, under the reduced pressure produced by a water pump, to a final volume of 15 ml. (It is important that this evaporation be carried to the same volume each time.) Twenty ml. of 95% methanol and 5 ml. of a saturated aqueous solution of potassium hydroxide are added to the residue in the flask, and the mixture is heated under reflux for 10 min. The sample is cooled immediately; 40 ml. of water are added and the contents are then transferred to a 500 ml. separatory funnel. The flask is rinsed with an additional 40 ml. of water, followed by rinsings with two 25 ml. portions of ether, which are also added to the contents of the funnel. The methanol-water solution of vitamin A is then extracted with 25 ml. portions of ether until the ether layer is colorless. It has been shown that the absence of yellow color in the ether phase is an indication of complete extraction of vitamin A.

The ether extract is washed with water until the wash is neutral to litmus, and then dried over anhydrous sodium sulfate. The extract is filtered through sodium sulfate into a volumetric flask. A crystal-clear, yellow filtrate should result.

Spectrophotometric Estimation of Vitamin A. The sample is made up to volume (250 ml.), and its optical density is determined on a Beckman spectrophotometer (43) at 326 mμ for vitamin A and 450 mμ for total yellow color. Fifteen per cent of the density at 450 mμ is subtracted from the density at 326 mμ as a correction for absorption at 326 mμ due to the yellow pigments present. The vitamin A present may be calculated by means of the following equation:

$$\gamma \text{ of vitamin A/g.} = \frac{D326 - 15\%\ D450}{0.176} \times \frac{\text{volume in ml.}}{\text{weight in g.}}$$

A comparison of the results obtained by this method with those obtained in the bioassay, showed only fair agreement.

Since the advent of vitamin enriched foodstuffs, *etc.*, the necessity for a control assay procedure has been obvious. Oser, Melnick and Pader

(115) have shown that the direct spectrophotometric assay of vitamin A frequently gives erroneously high results, even when an unfortified sample of the product is available for control purposes. These high results are due to the presence in the product of large amounts of extraneous material, even in the unsaponifiable portion, which absorb in the same region of the ultraviolet spectrum as vitamin A.

Where the direct spectrophotometric method has given high results, the absorption curves of the unsaponifiable fractions deviate markedly from the characteristic curve of vitamin A (115).

The use of unfortified blank samples in the direct assay increases the specificity, but such samples are not always available. The increase in specificity probably involves a decrease in the precision of the method.

The modified spectrophotometric procedure (116, 117, 118) which obtains a blank by destructive irradiation of the vitamin A in part of the samples does away with the necessity of having an unfortified control sample.

(2) *The Destructive Irradiation Method*

In 1926, Peacock (26) pointed out that vitamin A was destroyed by irradiation. This observation suggested to Morton and Heilbron (27) that vitamin A might show selective absorption in the ultraviolet region of the spectrum. These authors showed that the extinction coefficient of the 328 mμ maximum of vitamin A concentrates decreased at the same rate as the "chromogenic power" toward antimony trichloride, when solutions were irradiated in silvered-quartz vessels. Animal experiments carried out at the same time showed that the decrease in the extinction coefficient at 328 mμ corresponded roughly to the decrease in vitamin A content. Morton and his colleagues (30, 31, 112) confirmed the correlation between the extinction coefficient and the biological potency by more quantitative animal experiments.

Demarest (116) pointed out that the validity of a method of vitamin A assay based on destructive irradiation would depend on 3 assumptions.

(1) Irradiation destroys vitamin A and the end product has no appreciable absorption at 328 mμ.

(2) Other absorbing substances present will not undergo appreciable change in their absorption at 328 mμ during irradiation.

(3) Irradiation causes quantitative destruction of vitamin A.

There has been some controversy as to whether or not these conditions can be fulfilled (20, 119). Three procedures making use of the destructive irradiation procedure are given below in detail.

Little (120) has published a method which, when fully tried out, may get over some of the difficulties inherent in the spectrophotometric assay of vitamin A in low potency oils and animal tissues. The details of Little's method are as follows:

Apparatus. The absorption was carried out by means of a Hilger medium quartz spectrograph combined with a double disk rotating sector photometer, the source of radiation being a water-cooled, low-pressure, hydrogen discharge tube. The spectrograph slit was kept at a constant setting of approximately 0.09 mm.

The spectrograms were made using fused quartz absorption cells, similar to Hilger type C, and were recorded on 10 × 25 cm. (4 × 10 inch) photographic plates (Eastman 33), isodensity points being picked out by visual comparison with the aid of an opal-glass spectrum viewer and a small hand lens (4 ×).

Destructive irradiations were carried out with a Hanovia quartz mercury vapor arc, laboratory model, 110 volt, direct current, the lamp always being turned on 10 min. before irradiations were begun. The spectrograph cell containing the solution to be irradiated was placed in a small, water-packeted tin can (5 cm. deep, 5 cm. in diameter) which was fitted to be covered with the filter or filters desired. (Two filters were used: (1) red-purple Corex A (C.G. No. 986), 3 mm. thick and 5 cm. square; (2) an optical quartz cell, 5 cm. in diameter, containing 5 cc. of 0.2 *M* potassium hydrogen phthalate (121) resulting in a depth of solution of about 1 mm.) The can was raised into the hood of the lamp during the irradiation, so that the cell being irradiated was about 18 cm. from the mid-section of the arc.

Materials. *Cyclohexane.* A special grade was obtained (Eastman No. 702) and redistilled before use. In cases where the cyclohexane contained benzene or other impurities, it was purified by treating with fuming sulfuric acid. Each new or purified lot of solvent was compared spectrographically with distilled water and with the last solvent used.

Ethyl Alcohol. Ethanol 95% was treated with silver nitrate and potassium hydroxide, and the filtered solvent was then refluxed with *m*-phenylenediamine dihydrochloride and distilled.

Wet Ether for Extractions. Anhydrous ether was shaken periodically with about ⅛ its volume of 5% aqueous potassium hydroxide for 2-3 hr. and distilled slowly from the alkaline solution. This solvent was always purified less than 24 hr. before use.

Dry Ether. Anhydrous ether was distilled onto sodium wire and used as required from the storage bottle.

Procedures. *Unsaponifiable Fraction of Reference Oil.* The procedure of Wilkie (122) was used with the following modifications; a portion of 300-400 mg. of oil was taken for a sample and refluxed for 10 min. with 5% alcoholic potassium hydroxide. Following the ether extractions, 100 cc. of water were poured through the combined ether extracts without agitation. The water layer was drawn off and the ether solution washed with 3-5 cc. of water, with vigorous shaking. The resulting emulsion was broken by a final wash with 100 cc. of water, the water again being poured in without agitation. The washed ether extract was filtered by suction through a layer of anhydrous sodium sulfate on a sintered-glass funnel into a ground-glass vacuum distillation apparatus. The filter and anhydrous sodium sulfate were washed with two small portions of anhydrous ether, and the solvent was removed at room temperature, under reduced pressure, and in an atmosphere of nitrogen. The cyclohexane was introduced by means of a small, side-arm separatory funnel before the vacuum was broken. The cyclohexane solution was then transferred to a volumetric flask and made up to volume. The remaining air was displaced with nitrogen and the spectrographic assay run within 24 hr.

As was pointed out by McFarlane and Sutherland (123) vaseline should not be employed as a stopcock lubricant in the separatory funnels. Glycerol was used in this work and found to be satisfactory.

Unsaponifiable Extract of Animal Tissues. The animal tissues were dissected out, placed in a weighed cork-stoppered 50 cc. Erlenmeyer flask, re-weighed, and covered with 5% aqueous potassium hydroxide. (Care was taken to remove all fatty tissue, and the tissues were blotted carefully with paper toweling to remove excess blood.) The air in the flask was displaced with nitrogen, and the tissues were stored in the dark under refrigeration until assayed.

On removal from storage, the flasks were placed in a water bath at 80° C. for 5 min. or until complete dissolution occurred, cooled, and the tissue solution was transferred to a 50 cc. separatory funnel. The solution was shaken with 5 cc. of ethanol, followed by 25 cc. of wet ether. [Davies (124) found that a preliminary shaking with alcohol makes it possible to extract 99% or more of the vitamin A with a single ether extraction.] The water layer was drained into a second separatory funnel and again extracted with 25 cc. of wet ether. The ether extracts were then combined in a reflux flask with a boiling chip, and the ether was boiled off in a 60° C. water bath.

The residue was cooled, 25 cc. of ethanol and 3 cc. of 50% aqueous potassium hydroxide were added, and the mixture was refluxed in a water bath for 10 min., using a ground-glass reflux. This solution was cooled, 30 cc. of water were added, and the whole was transferred to a 250 cc. separatory funnel. From here on the sample was treated as given above for the unsaponifiable fraction of a cod liver oil.

Solutions of muscle tissue prepared in this manner were always clear and colorless but became somewhat turbid when irradiated. Occasionally this turbidity formed when the prepared solution had to be stored in the refrigerator overnight, in which case filtering into the absorption cells solved the difficulty. The following step, however, invariably led to a clear, colorless final solution with no tendency to become turbid either on refrigeration or irradiation.

After saponification, the sample was cooled, 30 cc. of water and 10 drops of saturated sodium sulfate were added, and the flask was allowed to stand 5-10 min. in an ice bath. The solution was then filtered (Whatman No. 44) into a separatory funnel and the flask and filter were washed with a few cc. of a mixture of half 95% ethanol and half water, saturated with sodium sulfate. The extraction was then carried out as usual.

From the applications of this treatment to preparations of muscle tissue, liver tissue, and cod liver oil, it appears that such a "salting out" does not cause any appreciable loss of vitamin A.

In the course of this work, specific extinction coefficients were determined by three different methods—(1) direct measurement, (2) irradiated measurement, and (3) modified photometry.

The direct measurement is generally used in spectrographic work, in which case, the spectrogram is taken with one absorption cell containing the solution under test and the other cell filled with pure solvent. The specific extinction coefficient is represented by the symbol $E^{1\%}_{1\,\mathrm{cm.}}$.

In the second case, two spectrograms are taken; one a direct measurement as above, and the other a measurement of the same preparations left in the cells, the solution having been irradiated. In this case, the symbol $E^{1\%}_{1\,\mathrm{cm.}\ (\mathrm{irrad.})}$ is used, where

$$E^{1\%}_{1\,\mathrm{cm.}\ (\mathrm{irrad.})} = E^{1\%}_{1\,\mathrm{cm.}\ (\mathrm{original})} - E^{1\%}_{1\,\mathrm{cm.}\ (\mathrm{residual})}$$

The method of modified photometry as suggested by De (125) requires but one spectrogram, in which case both cells are filled with the solution under test, the solvent cell being irradiated before the spectrogram is taken. The symbol $E^{1\%}_{1\,\mathrm{cm.}\ \mathrm{mod.}}$ will be used in this case.

It is obvious that methods 2 and 3 should give the same specific extinction coefficient for any given solution and also a measure of those constituents of the solution which are destroyed by irradiation—*i.e.*, vitamin A if the effect can be made specific for the vitamin.

Filters for Use in the Destructive Technique. In order to cause as little destruction of the absorbing impurities as possible, it is necessary to cut down the radiation of the mercury arc. All radiations between 200 and 300 mμ should be eliminated, whereas those between 300 and 400 mμ should be as strong as possible. In fish oils, the absorbing impurities absorb at two main maxima 230 and 270 mμ, and in low potency oils these maxima overshadow the vitamin maximum.

Saunders (121) first suggested the use of a solution of potassium acid phthalate as a filter for ultraviolet light. Such solutions cut out completely all radiations between 200 and 300 mμ. Little (120) in his destructive irradiation assay of vitamin A has used two filters (1) red-purple Corex A, CG No. 986 and (2) CG No. 986 plus 1 mm. thick layer of 0.2 *M* potassium acid phthalate solution contained in a quartz optical cell. Little states that little change occurred in the absorption of the extraneous material upon irradiation in the majority of solutions of vitamin A preparations used. However, in the case of vitamin A preparations from muscle tissue and low potency liver tissue the combination filter (2) was required in order to obtain specificity. Little states "Filter 2 might well be used for all samples regardless of potency."

The absorption curves for these two filters are given in Fig. 5.

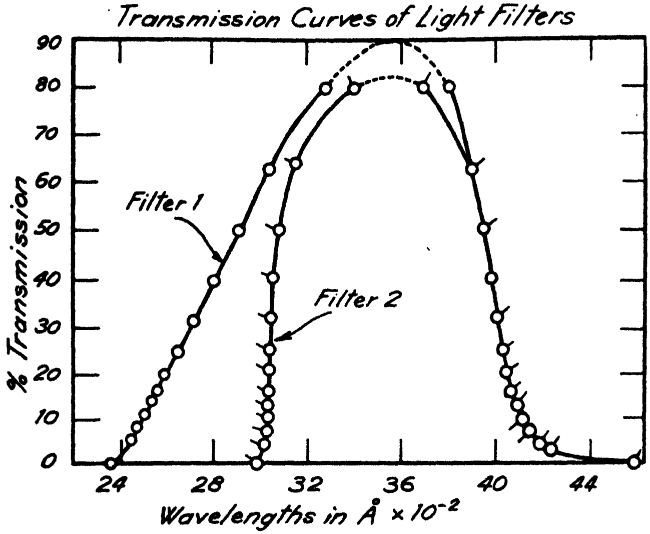

Fig. 5. Transmission curves of light filters. (1) red-purple Corex A, CG#986, (2) CG#986 plus I mm. thick layer of 0.2 *M* potassium acid phthalate solution (after Little (120)).

The biologic assays of Neal, Haurand and Luckmann (117), on unsaponifiable fractions before and after irradiation have demonstrated the complete loss of vitamin A potency paralleling the loss of absorption at 328 mμ.

The curves obtained by Little's method satisfy two of Demarest's

TABLE X

Direct and Irradiated Measurements of Vitamin A Potency of Concentrates, Fish Liver Oils, and Oil Dilutions

	Whole oil				Unsaponifiable fraction			
Oil No.	Known potency	Potency direct	Potency irrad.	Difference, direct and irradiated	Potency direct	Potency irrad.	Difference, direct and irradiated	Difference, whole oil and unsaponifiable irradiated
		I. U./g.		*Per cent*	I. U./g.[a]		*Per cent*	*Per cent*
1	260,000	237,000	237,000	0	225,000	225,000	0	6
2	60,000	72,500	72,500	0	60,000	60,000	0	21
3	2,400	2,750	2,400	14	2,500	2,500	0	4
4[b]	1,700	2,020	1,700	19	1,875	1,670	12	2
5[c]	240	450	275	64	325	240	35	14
6[d]	46	250	87	187	140	70	100	38

[a] I. U./g. of original oil.

[b] U. S. P. oil #2.

[c] Oil #3 diluted 1: 10 by weight with corn oil.

[d] Oil #3 diluted 1: 50 by weight with corn oil.

(116) conditions, *viz.*, (1) and (3). The absorption at 328 mμ is completely destroyed in the case of the liver fractions and the crystalline vitamin indicating 100% destruction of the vitamin.

Condition (2), *i.e.*, the irradiation produces no appreciable change in the absorption at 328 mμ of substances present other than vitamin A, is harder to satisfy than the others. Little says on this point, "The specificity of the irradiation for the vitamin is well demonstrated" by the curves obtained on various preparations of fish oils and tissue extracts provided filtered light is used.

The value of this method for the assay of vitamin A over a wide range of potency is shown in Table X.

It will be seen from the table that this technique can be applied with a fair degree of accuracy to whole oils of much lower potency than 10,000 I.U./g., which is the potency above which it is a common practice to assay directly by the direct spectrophotometric method. Similarly, lower potency unsaponifiable preparations can be assayed by this technique than can by the normal assay.

Many spectrophotometric methods have been put forward for the estimation of the total vitamin A (*i.e.*, vitamin A and carotene) content of dairy butters apart from the biological assay, which is of limited accuracy (76).

Gillam *et al.* (126) separated the carotene and xanthophyll and determined these separately by spectrographic means. The vitamin A was determined by means of its absorption at 328 mμ in the presence of carotene in one fraction and the presence of xanthophyll in another. This method does not take into account the naturally occurring non-vitamin A impurities which have general absorption in the region of 328 mμ, and further, it is a somewhat laborious method.

Gillam (127) improved this method by showing that the carotene accounts for 94% of the light absorption at 455-460 mμ in chloroform solution. He further showed that the vitamin A absorption at 328 mμ could be corrected by a constant factor to account for the absorption at this wavelength due to carotene and xanthophyll. This method does not take into consideration the absorbing non-vitamin A impurities. Baumann and Steenbock (128) introduced the step of freezing out the absorbing impurities from the unsaponifiable fraction.

Fraps and Kemmerer (129) have estimated the vitamin A content of butter by measuring the absorption at 328 mμ of a methanolic solution of the unsaponifiable fraction after the freezing out of absorbing impurities. The carotene content was estimated separately by colorimetric comparison with potassium dichromate. This method tends to give high results.

Neal, Haurand and Luckmann (117) have put forward a method based on the destruction of the vitamin A and carotene in a portion of the unsaponifiable fraction by ultraviolet irradiation. This irradiated portion is used as a control for the simultaneous spectrophotometric estimation of vitamin A and carotene. Their method is as follows:

Spectrophotometer. Adam Hilger, Ltd., intermediate quartz spectrograph with Spekker photometer, equipped with tungsten steel electrodes as a source of light.

Quartz absorption cells. Hilger Type C, 1-cm. quartz Kjeldahl-shaped flasks, 25 cc.

Ultraviolet lamp. Mercury lamp, rectifier-type, quartz, are operated to be equivalent to a 250 watt direct current Uviarc: Uviarc poultry treater, Type RT, Spec. 100, Cooper Hewitt Electric Co., Hoboken, N. J. Operated through an alternating current auxiliary, Code No. 260, 7A4X 4, 186-254 volts, 60 cycles, 2.4 amperes, 450 watts, made by Cooper Hewitt Electric Co., Hoboken, N. J. (The spectral radiation for this lamp can be obtained by taking three-fourths of the values given in column 1—*i.e.,* for the 120-volt direct current Uviarc—of data sheet S-201 compiled by the Commercial Engineering Department, General Electric Vapor Lamp Co., Hoboken, N. J.).

Extraction of Unsaponifiable Material. Melt the butter in a water bath at about 60° C., and separate the fat by filtration through a Whatman No. 12 folded filter paper.

Saponify 25 g. of the fat with 30 cc. of a 20% solution of KOH in alcohol (SD#30) by boiling, with a suitable reflux arrangement, for 5 min. only.

Dilute the alcoholic solution with water to approximately 4 volumes, and cool in an ice-water bath. Then extract the unsaponifiable matter with cold ethyl ether (usually 4 extractions with the following successive amounts of ether are sufficient: 180, 150, 100, and 50 cc.). Vigorous shaking is required for complete extraction of the unsaponifiable material.

Composite the ether extracts and wash with 150 cc. portions of distilled water until substantially free of alkali, taking due care to avoid troublesome emulsions. Usually 6 washings are sufficient. The first two washings should be made by merely pouring the water through the ether without shaking.

Filter the ether solution through filter paper and concentrate to 25-50 cc. by distillation on a steam bath. Remove the remainder of the ether by evaporating on a steam bath, under a stream of carbon dioxide to prevent oxidation. When substantially all the ether has been evaporated, cool immediately to about 21° C. (70° F.), and dissolve the unsaponifiable residue in cyclohexane. Make the cyclohexane up to 50 cc. (50% solution, weight to volume, on original oil basis). Filter and store in the dark at about 4-10° C. (40-50° F.) until the sample is examined spectrophotometrically. (The sample should not be stored more than 2 days before its vitamin content is determined.)

Note. Both the ether and alcohol must be carefully purified immediately before using. This is satisfactorily accomplished in the case of ethyl ether (anhydrous C.P.) by letting the ether stand with intermittent shaking, for 3 hr. over a 5% aqueous KOH solution, then slowly distilling. The alcohol (SD#30) is satisfactorily purified by treating with KOH and aluminum grains followed by distillation (130).

Spectrophotometric Determination of Carotene and Vitamin A. Divide the cyclohexane solution of unsaponifiable material in two parts, and irradiate one portion, contained in a 25 cc. Kjeldahl-shape quartz flask, under the Uviarc as follows (allowing at least 10 min. for the lamp to come to full operating temperature before starting irradiations): Stopper the flask with a cork wrapped in aluminum foil, and support the flask in such a position that the cork rests against the rim of the lamp and the bulb of the flask is held about 10 cm. (4 in.) away from the mercury tube of the lamp. Place a sheet of aluminum foil about 7.5 cm. (3 in.) below the flask being irradiated (to reflect the light back toward the sample). Agitate the sample every 15 min. by gently tapping the flask (for example, with a pencil). Allow the sample to heat up as much as the lamp will heat it, provided that the temperature of the sample does not reach the boiling point of cyclohexane.

Irradiate until the vitamin A and carotene have been destroyed. Under the author's conditions, 1-1.5 hr. has proved sufficient time for irradiation. Destruction of carotene and vitamin A can be estimated by the disappearance of the carotene yellow color, and by the Carr-Price test.

After cooling to about 21° C. (70° F.), filter the irradiated solution, which must be clear and colorless, and determine carotene and vitamin A in the non-irradiated solution by means of the spectrophotometer (1 cm. cells) using as a control the ultraviolet irradiated solution. Expose the plates at density settings ranging from 0 to 1.50 in increments of 0.05, with the exposure time graduated up to about two seconds on Eastman 33 plates (D 72, diluted 1:2, is a satisfactory developer for these plates).

Read the carotene and vitamin A match points from the same plate. The vitamin A is read at 328 mμ (3280 Å) and the carotene at 460 mμ (4600 Å). (These wave lengths, as points of maximum absorption, are in agreement with published works (127), and have been verified in this laboratory for solutions of vitamin A concentrates and carotene in cyclohexane.) Of course, the difference in absorption between the non-irradiated sample, at the wave lengths indicated above, is a measure of the vitamin A and carotene content of the sample.

Calculations. **A.** Carotene in Terms of Vitamin A.

Match point at 4600 Å $\times$ 0.94 = corrected match point at 4600 Å.

$$\frac{\text{Corrected match point at 4600 Å.} \times 4.54 \times 1000}{0.50 \times 2.1 \times 0.6} =$$

U.S.P. XI vitamin A units/lb. of butterfat,

due to carotene, based on: (1) 94% of the light absorption at 4600 Å. is due to carotene (127), (2) $E^{1\%}_{1\,\text{cm.}}$ at 4600 Å. for carotene in cyclohexane = 2100. (established for the author's instrument by the use of a sample of β-carotene containing about 10% of α-carotene, obtained from the S.M.A. Corp., Cleveland, Ohio), (3) By definition, 0.6 γ of β-carotene equals one International Unit of vitamin A (45).

B. Vitamin A.

$$\text{Match point at 3280 Å.} - \frac{\text{match point at 4600Å.}}{6.5} = \text{corrected match}$$

point at 3280 Å.

$$\frac{\text{Corrected match point at 3280 Å.}}{0.50} \times (2140 \times 4.54) = \text{U.S.P. XI vita-}$$

min A units/lb. of butterfat, due to vitamin A, based on: (1) The absorption at 328 mμ due to carotene plus xanthophyll is given by dividing the observed value of $E^{1\%}_{1\,\text{cm.}}$ for these substances at 455-460 mμ (127) by the factor 6.5; (2) 2140 as the instrument factor for vitamin A. *Total* I + II = total U.S.P. units of vitamin A/lb. of butterfat due to both carotene and vitamin A. The results obtained by this method were found to be in reasonable agreement with the U.S.P. XII biologic assay.

There is little literature on the subject of the spectrophotometric method for the assaying of vitamin A in margarine, and it was not until the promulgation of the federal definition (131) and standard of identity for oleomargarine that there was real necessity for such a method. Neal and Luckmann (132) have developed a method based on the spectrophotometric determination of the vitamin A in the unsaponifiable fraction and irradiating a fraction of this material for use as a control.

The method these authors had used for butter (117) was not applicable to oleomargarine, due apparently to the fact that the unsaponifiable fraction of vegetable oils from which margarine is prepared differs from that of butterfat. Butterfat is more readily saponified than margarine.

These authors stress the purity of the solvents used in the assay. They "have found either cyclohexane or methyl cyclohexane, specified as 'purified for spectrophotometric use and free of extraneous ultraviolet absorption' and obtained from Eastman Kodak Company, Rochester, N. Y., to be satisfactory in most instances. Vitamin A is stable in either of these solvents for several days, provided that the solutions are stored in the dark, and the solvents themselves are sufficiently pure."

"It has been the authors' practice to verify the suitability of each lot of either cyclohexane or methyl cyclohexane by spectrophotometric comparison with a sample of known purity. A sample is considered usable only if it shows no extraneous absorption in the region between 5000 and 2200 Å.; extraneous absorption within this range is considered evidence of impurities and the material is rejected. A method satisfactory for the purification of cyclohexane containing a small amount of impurities has been given, but is not satisfactory for the purification of methyl cyclohexane.

"The ether and alcohol used for extracting the unsaponifiable material must be especially pure and free of peroxides, in order to avoid oxidation of the extracted vitamin A. These solvents must be carefully purified before use, even though the best grades are purchased. A method for the purification of anhydrous C.P. ethyl ether was given in a previous publication (117). Specially denatured No. 30 alcohol can be satisfactorily purified by an A.O.A.C. method (130)."

In their assay, Neal and Luckmann (132) used the same equipment as Neal, Haurand and Luckmann in their assay of vitamin A in butter (see above). They also used a Beckman quartz spectrophotometer, Model D, with Corex absorption cells (1 cm. square).

The Hilger and the Beckman instruments gave equally satisfactory results.

The method is as follows:

Extraction of Unsaponifiable Material. Melt the margarine in a water bath at about 60° C., and separate the fat by filtration through a Whatman No. 12 folded filter paper (or other equivalent paper).

Saponify 20 g. of the separated and filtered fat with 30 cc. of alcoholic KOH (200 g./l. of specially denatured No. 30 alcohol) by boiling, with suitable reflux arrangement, for 15 min.

Dilute the alcoholic solution with water to approximately 4 volumes and cool in an ice-water bath. Extract the unsaponifiable material with cold ethyl ether. At least 6 extractions, with the following successive amounts of ether, are required for complete removal of the unsaponifiable material: 200, 150, 100, 50, 50, and 50 cc. (Both the ether and alcohol must be carefully purified immediately before use. This is essential for dependable results.) Vigorous shaking is also necessary for complete removal of the unsaponifiable material. The sample should be adequately protected from sunlight during extraction.

Composite the ether extracts and wash with 150 cc. portions of distilled water until substantially free of soap. (There should be no appreciable turbidity developed after acidifying the wash water with 10% HCl.) Adequate precautions should be taken to avoid troublesome emulsions; the first two water washes should be made by merely pouring the water through the ether without shaking.

Filter the ether solution through filter paper and concentrate to 25-50 cc. by distillation on a steam bath. Remove the remainder of the ether by evaporating, on a steam bath, under a stream of carbon dioxide to prevent oxidation. When substantially all the ether has been evaporated, cool immediately to about 20° C. (70° F.), and dissolve the unsaponifiable material in optically clear cyclohexane or methyl cyclohexane. Make a solution up to 50 cc. (40% solution weight to volume, on original fat basis). Filter and store in the dark at about 4-10° C. (40-50° F.) until the sample is examined spectrophotometrically, but not longer than 48 hr.

Spectrophotometric Determination of Vitamin A. Divide the cyclohexane (or methyl cyclohexane) solution of unsaponifiable material into two parts, and irradiate one portion under the Uviarc as follows: Transfer the solution to be irradiated into a 25 cc. Kjeldahl-shaped quartz flask, and stopper the flask with a cork wrapped in aluminum foil. Allow at least 10 min. for the lamp to come to full operating temperature before starting irradiation.

Support the flask in such a position that the cork rests against the rim of the lampshade (the lamp in use is equipped with a shade 21 cm., 14 in., in diameter) and the bulb of the flask is held about 10 cm. (4 in.) away from the mercury tube of the lamp. Place a sheet of aluminum foil about 7.5 cm. (3 in.) below the flask being irradiated, in order to reflect the light back toward the sample. Agitate the sample every 15 min. by gently tapping the flask, for example, with a pencil. Allow the sample to heat up as much as the lamp will heat it, provided that the temperature does not reach the boiling point of the solvent being used.

Irradiate until the vitamin A has been destroyed. Under the author's conditions, approximately 2.5 hr. have been required for complete destruction of the vitamin A contained in this concentration of margarine fat unsaponifiable. Destruction of vitamin A can be estimated by the Carr-Price test, and the time of irradiation required for the ultraviolet lamp in use can be established spectrophotometrically by irradiating until there is no further decrease in absorption of the irradiated sample at 3280 Å.

After cooling to about 21° C. (70° F.), filter the irradiated solution, which must be clear and colorless, and determine vitamin A in the non-irradiated solution by means of the spectrophotometer (1 cm. cells), using the ultraviolet-irradiated solution as a control. With the Hilger spectrophotometer, expose the plates at density settings ranging from 0 to 1.50 in increments of 0.05, with the exposure time graduated up to about 2 sec. on Eastman No. 33 plates. These plates are satisfactorily developed with Eastman D72, diluted 1:2. Considerable time can be saved, without any sacrifice in accuracy, by using a Beckman spectrophotometer. For this instrument, 1 cm. square type Corex absorption cells are satisfactory. A tungsten lamp instead of a hydrogen discharge tube can be satisfactorily used as a source of ultraviolet light.

Read the match point (or density) at 3280 Å., and calculate the $E^{1\%}_{1\,\text{cm.}}$ value of the sample under test. The difference in absorption at 3280 Å., between the non-irradiated sample and the irradiated sample, is a measure of the vitamin A content of the sample.

This method has been successfully used for determining the vitamin A content of a variety of domestic vegetable oil margarines, but no attempts have been made to apply the method to animal fat margarines or to coconut oil type margarines.

Calculations.

$$\frac{\text{Match point (or density) at 3280 Å.}}{40} \times 2140 \times 454 = \text{U.S.P. units of}$$

vitamin A/lb. of margarine fat.

$$\frac{\text{\% fat in sample} \times \text{U.S.P. units of vitamin A/lb. of butter fat}}{100} =$$

U.S.P. units of vitamin A/lb. of margarine.

These calculations are based on 2140 as the conversion factor for vitamin A.

Derivation of Conversion Factor of 2140. The conversion factor to be used for converting from $E^{1\%}_{1\,\text{cm.}}$ value at 3280 Å., to U.S.P. units of vitamin A/g. of oil, was determined for the instruments employed by use of the U.S.P. standard of reference cod liver oil. The method consisting of $E^{1\%}_{1\,\text{cm.}}$ value determinations, made by the instrument being calibrated, on the unsaponifiable fraction of U.S.P. standard of reference cod liver oil containing 3000 U.S.P. units/g. of oil, has been described (117). The unsaponifiable material from the reference cod liver oil, for spectrophotometric study, is a modification of the procedure published by Wilkie (133).

Table XI shows the results of conversion factor determinations made with the Hilger spectrophotometer, using the current U.S.P. standard of reference cod liver oil, containing 1700 U.S.P. vitamin A units/g. of oil. These determinations include results obtained by the use of both cyclohexane and methyl cyclohexane as solvents. In order to minimize the effect of any possible instability of the U.S.P. reference oil, fresh or practically fresh samples of the reference oil were used for each determination as indicated.

TABLE XI

Establishment of Conversion Factor (Hilger Spectrophotometer)

Date of determination	Per cent solution (original oil basis)	Solvent	Match point at 3280 Å.	$E^{1\%}_{1\,\text{cm.}}$ Value	Conversion factor
2/28/41	1.000	Cyclohexane	0.795	0.795	2138
2/28/41	1.000	Methylcyclohexane	0.795	0.795	2138
6/12/42	1.000	Methylcyclohexane	0.790	0.790	2152
8/4/42	1.000	Methycyclohexane	0.790	0.790	2152
8/4/42	1.000	Methylcyclohexane	0.800	0.800	2125
8/18/42	1.000	Methylcyclohexane	0.795	0.795	2138
					Av. 2137

Conversion factor of 2140 is used. Similar results were obtained by use of Beckman spectrophotometer.

Determinations dated 2/28/41, 6/12/42, and 8/4/42 were made on fresh samples of reference oil, not previously opened. Determination dated 8/18/42 was made on same sample used on 8/4/42 after storage for 2 week interim at 7° C. (45° F.) in the dark and under an atmosphere of carbon dioxide.

3. PROVITAMINS A

A. GENERAL

Many difficulties beset the quantitative estimation of the provitamin A in natural materials. In materials of plant origin, the task is somewhat simplified by the absence of vitamin A which occurs only in products of animal origin.

The only accurate method of assaying provitamins A is the biological method, which gives values for the total carotenoid mixture expressed as β-carotene equivalents, when β-carotene is used as a standard reference compound.

The carotenoids which show vitamin activity have been listed in Table III. The percentage provitamin A activity of various carotenoids are given in Table XII based on all-*trans* β-carotene = 100.

The physical methods for determining the provitamins A are dependent on their separation from the other non-active carotenoids present.

TABLE XII

	Carotenoid	Per cent provitamin A activity	Ref.
All-*trans*.	α-carotene	53	134
	Neo-α-carotene U	13	134
	Neo-α-carotene	16	135
All-*trans*.	β-carotene	100	134
	Neo-β-carotene B	53	135
	Neo-β-carotene U	38	134
All-*trans*.	γ-carotene	28	136
	Pro-γ-carotene	44	136
	Cryptoxanthin	57	137

Two general methods have been developed for the quantitative estimation of carotene in plant pigments. These two approaches stem from the methods of Tswett (138) and Willstätter and Stoll (139). The former used an absorbing column, through which a solution of the plant pigments was passed, and thus obtained a chromatographic separation of the chromogens. Willstätter used a two-phase solvent distribution technique, in order to separate the carotenes from the xanthophylls and the chlorophylls.

In more recent times, a combination of these two methods is more generally used, particularly for complicated mixtures.

The provitamins A may be present in natural material, at least in part, in a bound form, as esters or protein complexes. They may be separated from such complexes by simply heating to 40-60° C. In natural material, particularly animal tissue, where the hydroxylated carotenoids may be present in the form of esters, the free pigments may be obtained after being saponified by means of alcoholic KOH at room temperature, or with the application of heat. Kuhn and Bielig (140) have used quaternary ammonium salts, such as lauryldimethylbenzylammonium bromide to effect the freeing of combined carotenoids. After such treatments, the carotenoids can be readily extracted from the source material.

Many solvents and mixtures of solvents have been used for the extraction of the provitamins A from such prepared samples, *e.g.*, alcohols, acetone, ether, diacetone alcohol, petroleum ether, hexane, and petroleum-ether-methanol or petroleum ether-acetone mixtures. Petroleum ether is the most favored extractant for this purpose. In most cases, the best extraction of the carotenoids from plant material, *etc.*, is obtained by agitation of the material and extraction in a Waring Blendor or grinding the sample with quartz sand in the presence of the extractant.

Petering, Wolman and Hibbard (141) have introduced a method designed to separate the chlorophyll from the carotenoids. The plant tissue, finely cut, is extracted by grinding repeatedly with quartz sand and acetone until no more pigments are extracted. The combined extracts are made up to volume and an aliquot is boiled with a small amount of activated barium hydroxide or barium hydroxide octahydrate. The barium hydroxide adsorbs the chlorophyll and may be separated from the soluble pigments by filtration. The xanthophylls and flavones can be removed by the usual methods from the acetone filtrate leaving the carotenes.

Benne *et al.* (142) have compared the barium hydroxide method, and some of its modifications with other methods of carotene determination and found them to be comparable. Peterson (143) has pointed out some precautions that should be observed in this method.

B. THE PHASIC SEPARATION

The most common method of preliminary separation of the biologically active carotenes from the non-active pigments depends on the distribution of the components between two immiscible solvents. The solvents most generally employed are light petroleum, or light petroleum mixed with ether, as the upper or "epiphasic" layer and aqueous methanol (85%-95%) or aqueous diacetone alcohol as the lower or "hypophasic" layer. The different categories of carotenoids can be separated quantitatively by repeated distribution between such solvents.

The carotenoid hydrocarbons are *epiphasic* whereas the free carotenoid alcohols and the hydroxyketones are *hypophasic.*

The carotenoid alcohols, in the form of esters, are epiphasic, but after saponification with methanolic KOH become hypophasic, thus a separation of the esterified from the non-esterified carotenoids can be effected.

Borodin, in 1883 (144), first showed that the carotenoid pigments could be separated into two fractions (a) a petroleum ether-soluble fraction and (b) an alcohol-soluble fraction.

Willstätter and Stoll (139, 145), in 1913, published a method for the determination of carotene, which has been the basis of many of the methods developed since then.

These authors ground the fresh plant material with quartz sand, in a mortar, in the presence of 40% acetone. The ground material is then washed with 30% acetone until the filtrate is colorless, and finally with pure acetone. The combined aqueous acetone extracts are treated with ether and saponified with methanolic KOH. The ether solution is then washed with water to remove acetone and methanol, and the ether removed. The residue is taken up in petroleum ether, and this solution is then freed from xanthophylls by extraction first with 85% methanol, then 90% methanol, and finally with 92% methanol, until the washings are colorless. The xanthophylls are recovered from the combined extracts by extraction with ether. Both the petroleum ether and the ether solutions are freed from methanol by washing with water, dried and made up to volume and the concentrations determined colorimetrically by comparison with a 0.2% solution of potassium dichromate.

Many modifications of this basic method of separation have been made. Coward (146) carried out all the steps in an atmosphere of nitrogen. Kuhn and Brockmann (147) extracted the dried plant material with a mixture of petroleum ether and absolute methanol and then diluted the extract with water to give two phases. The petroleum ether phase is then extracted with 90% methanol. The esters which may be present in the petrol phase are then saponified with 5% KOH in ethanol at 40° C. and the hydroxylated carotenoids are extracted with 90% methanol from the petrol phase.

In 1934, Guilbert (148) published a modified method which was widely used for the determination of carotene. In this method, ether was used as the extractant. The pigments were transferred to petroleum ether and the esters saponified by boiling for 30 min. with saturated alcoholic KOH.

Peterson, Hughes and Freeman (149) shortened the Guilbert method and eliminated some manipulative losses. These authors used petroleum ether (b.p. 40-60° C.) in place of ethyl ether for the initial extraction of the sample.

Peterson (143) has since modified this method further, and he describes two alternative procedures for use with dry and fresh plant material, respectively. These two methods are quoted in detail:

Dry Plant Material. Weigh out the samples (1-5 g., more or less, depending on the relative potency), transfer to a 250 cc. Erlenmeyer flask, and add 100 cc. of freshly prepared, 10% ethanolic KOH. Fit the flasks with reflux condensers, and boil the contents on a steam bath or hot plate for 30 min. If portions of the sample collect on the sides of the flask, wash down with alcohol from a wash bottle. Cool the contents of the flask, then pour them into a sintered-glass filter funnel, applying a vacuum only until most of the solvent has come through. Then wash the residue alternately with 25 cc. portions Skellysolve B and absolute alcohol until the filtrate comes through clear. The suction should at no time be applied unless the sediment is partially covered by solvent. After the addition of each wash portion of solvent, more complete extraction may sometimes be obtained by stirring the sediment on the funnel plate with a stirring rod before applying suction. Transfer the filtrate to a 500 cc. separatory funnel.

Pour gently about 100 cc. of distilled water through the Skellysolve solution in the separatory funnel. Draw off the alkaline alcohol-water solution from the bottom of the funnel, and re-extract 3 times by shaking gently with 30 cc. portions of Skellysolve B using two other separatory funnels. Combine the Skellysolve extracts and wash free from chlorophyllins, flavones, alkali, and xanthophylls by shaking thoroughly with 30 cc. portions of 90% methanol (5 washes are generally sufficient) and re-extract the first methanol portion with 50 cc. Skellysolve B. Wash the Skellysolve B solution once with 50 cc. of distilled water to remove the alcohol and filter into a volumetric flask through filter paper upon which is placed a small amount of anhydrous sodium sulfate. After making the carotene solution up to a definite volume, determine the concentration by the spectrophotometer, photoelectric colorimeter, or colorimeter by comparison with 0.1% or 0.036% potassium dichromate.

Fresh Plant Material. Cut fresh plant tissue finely with shears and mix as thoroughly as possible. Weigh samples of 4-10 g. into a 250 cc. Erlenmeyer flask and proceed with digestion and washing as for dry materials. When the residue on the filter plate has been thoroughly washed, transfer it into a deep mortar with a well-defined lip, add 10 cc. of a saturated solution of KOH in ethanol, and macerate with the pestle. Add quartz sand and grind. From time to time wash down the sides of the mortar with a stream of alcohol from a wash bottle and grind until the tissue is fine. Transfer the contents of the mortar to the Erlenmeyer flask used in the original digestion, using more alcoholic KOH to effect the transfer. Add alcoholic KOH until the total volume is 100 cc., digest for 15 min., and proceed as indicated for dry tissue. Combine filtrates from both digestions and washings before extraction of xanthophylls.

The concentration of the aqueous alcoholic phase may play a considerable part in phasic separations, *e.g.*, with 95% methanol cryptoxanthin and rubixanthin are hypophasic, but become epiphasic with more dilute alcohol.

It has been known for some time that carotene extracts of plant material prepared by one of the modifications of the Willstätter-Stoll distribution method do not always agree in spectral properties with those of pure crystalline β-carotene (113, 143, 150, 151).

The value of $\log \frac{I_0}{I}$ at 450 mμ is very often too large with respect to that at 470 mμ (113). While $\log \frac{I_0}{I}$ at 480 mμ is generally less than that at 470 mμ.

The following table (XIII) compares the relative spectral absorptions at the 3 wave lengths of β-carotene and some typical carotene extracts. The values are expressed in percentages of absorption at 470 mμ.

TABLE XIII

	450 mμ	470 mμ	480 mμ
β-Carotene	118	100	105
Tall grasses	129	100	106
Egg yolk	117	100	92
Sorghum silage	123	100	100
Yellow corn	116	100	96

Hegsted, Porter and Peterson (152) developed a method which used aqueous diacetone alcohol to extract the petroleum ether extract of the carotenoids. Aqueous diacetone alcohol of 94% concentration has been recommended for this distribution (153, 154), and it has been shown (155) that it removes more non-carotene chromogens in the phasic separation than aqueous methanol. Zscheile *et al.* (154) have compared these two extractants in the case of 6 butterfats, and found that aqueous diacetone alcohol removes 6-9% more non-carotene pigments than aqueous methanol. The use of this extractant is particularly useful in the case of butters from cows fed acid or molasses silage, since such butters contain a much higher percentage of non-β-carotene chromogens. Haagen-Smit *et al.* (156) have used phosphoric acid (85%) to remove the xanthophylls from the petroleum ether extracts of plant tissue.

It would appear that there are certain limits to the purification of β-carotene that can be accomplished by phasic methods due to the similarities in partition coefficients of some of the impurities with those of β-carotene. In order to purify the carotene extracts further, resort must be made to chromatographic methods.

C. THE CHROMATOGRAPHIC SEPARATION

The evaluation of the vitamin A potency of carotenoid-containing materials is at the moment somewhat confused, due to the discovery, in 1940, that the epiphasic fractions of total carotenoids from a variety of sources contained *cis-trans* stereoisomers of the normal all-*trans*-β-carotene and other interfering pigments. This had been suspected for some time (113, 143, 151) on the basis of the abnormal absorption curves given by "carotenes" from different sources (Table XIII).

Beadle and Zscheile (22) have suggested the name "neo-β-carotene" for the main pigment impurity in the carotene fraction of plant materials.

Zechmeister (157) has published methods by which the carotenoids can be isomerized. Such "neo-β-carotenes" can be derived from β-carotene by a variety of methods, such as heating, iodine catalysis, acid catalysis, and the action of light. This author has proposed a system of nomenclature for such isomers (see also 158).

Two such stereoisomers of β-carotene have been found in extracts of plant material. Beadle and Zscheile (22) were able to separate two pigments from the petroleum ether extract of fresh spinach. These authors separated the chromogens contained in the petroleum extract by chromatographic analysis on activated alumina (Merck's anhydrous alumina according to Brockmann). Neo-β-carotene appeared immediately below the β-carotene band as a brownish-yellow zone. On elution (5% ethanol in hexane) the spectral characteristics of the β-carotene band were in complete agreement with those of the reference β-carotene standard. The lower band (neo-β-carotene), on elution, showed the spectral characteristics of the pseudo-α-carotene of Zechmeister and Tuzson (159) and Carter and Gillam (160).

Beadle and Zscheile (22) have found that the carotene extracts from many plants, which produce a homogeneous "β-carotene band" on magnesium oxide and had spectral characteristics differing slightly from those of pure β-carotene could be resolved into two zones when analyzed chromatographically on aluminum oxide.

The neo-β-carotene has only half the biological potency of β-carotene (135, 161). This isomer is a true *cis-trans* isomer of the normal all-*trans*-β-carotene.

Another interfering pigment in the epiphasic carotene fraction has been isolated by Kemmerer and Fraps (161) from alfalfa meal by chromatographic analysis on calcium hydroxide. This chromogen, "carotenoid X," has been shown (162) to be identical with the neo-β-carotene U of Polgár and Zechmeister (23).

Zechmeister and his colleagues (134) have evaluated the provitamin A activity of *neo-β*-carotene U and found it to have one-third of that of the all-*trans*-isomer. More recently Kemmerer and Fraps (163) have reported that *neo-β*-carotene U has about 25% of the biological activity of all-*trans-β*-carotene, but at the same time these authors have shown that this activity is due to steric rearrangement of part of the neo-β-carotene U into the all-*trans*-isomer and neo-β-carotene B in the digestive tract of the rats.

Many workers (161, 164, 165, 166) have found such petroleum ether-soluble pigments and have shown them to be without biological activity

It has been shown (165, 167) that solutions of β-carotene tend to isomerize on heating and even on standing. Fraps and Kemmerer (161) have shown that purified carotene, and purified carotene which had stood at room temperature with alcoholic potash, did not contain neo-β-carotene. However, when carotene was boiled with hexane or methanol, ethanol, or alcoholic potash the product contained 9.0, 10.4, 18.8 and 17.1%, respectively, of neo-β-carotene. From these results, it is essential that methods which do not involve heat be used in the extraction of carotene from plants.

Zscheile *et al.* (168) have proposed the following method to minimize the induced isomerism during the isolation of the carotenoids from butter fats:—

A butter sample from a cow fed a carrot-root carotene supplement was simply dissolved in ether, washed with water, dried with sodium sulfate, and filtered. The characteristic curve showed evidence of the presence of α-carotene and was very close to absorption curves of the ether extract after saponification, in spite of solvent differences due to the large amount of fat present. In this experiment, a 7 cm. thickness was studied spectroscopically by comparing an 8 cm. cell with a 1 cm. cell, both filled with the butter fat solution to avoid differences in apparent absorption due to refractivity differences.

Another butter fat was dissolved in hexane, and washed free of carotenols with 94% diacetone alcohol. The characteristic curve of the resultant carotene fraction corresponded well, between 4360 and 4950 Å, with that of a mixture of β-carotene and neo-β-carotene. Upon analysis for these two components, the percentage of β-carotene was 77.1 at 4780 Å and 79.1 at 4850 Å. Adsorption of this solution on columns of magnesia and alumina indicated the presence of two or more pigments.

The assay of provitamin A activity of most plant materials, either foods or fodders, depends ultimately on a colorimetric or spectrophotometric determination of the biologically active carotenoids. The complete separation of mixtures of biologically active and inactive carotenoids has been a major problem for many years.

A great deal of work has been done in order to find a method that would yield purer carotene extracts than those obtained by even the best phasic method. Most of these attempts have involved various types of selective adsorption.

The elegant method of chromatographic analysis introduced by Tswett in 1906 was given a fresh impetus in 1931 when Kuhn and Lederer (169) separated a crude carotene mixture into two isomeric hydrocarbons. Since then, the method of chromatographic analysis has been most valuable as a tool for the preparation of pure carotenoids and as an adjunct in the determination of the provitamins A.

The Tswett technique consists essentially of passing a solution of the pigments in petroleum ether or other suitable solvent down through an evenly packed column of an absorbent. The more strongly absorbed pigments remain near the top of the column and the less strongly absorbed pigments pass down the column as the chromatogram is developed by the continued passage of fresh solvent. The various pigments form bands in the column, the position of which is dependent on the number of conjugated double bonds, and the number of hydroxyl groups in the molecule. The various bands, after full development, are mechanically scraped out by means of a long thin spatula into separate beakers, and the individual pigments are removed or eluted from the absorbent by means of an eluant which, in the case of the carotenes, is usually petroleum ether containing 1-2% of alcohol. A full account of the techniques of chromatographic analysis, as applied to the isolation of pure carotenoids, may be found in the texts of Strain (97) and Zechmeister and Cholnoky (98) (*cf.* 170, 157).

In certain cases, the assay of the total hydrocarbon carotenoids is not sufficient, and it is desirable to ascertain the proportions of the various carotenes present in the mixture, since, for example, α-carotene shows only half the potency of the β-isomer. Again it may be necessary to assay the hydroxylated provitamin carotenoid, kryptoxanthin, in the hypophasic constituents of the total carotenoid mixture. For such determinations, the use of chromatographic analysis is obligatory for the quantitative separation of individual carotenoids. It should be pointed out, however, that artefacts may arise during the separation, which yield misleading results.

The absorption maxima and the extinction coefficient of the carotenoids vary from solvent to solvent. (See Tables III and XV.) The absorption curve of β-carotene is shown in Fig. 6.

Many different absorbents have been used for the chromatographic analysis of carotenoid extracts. Fraps and his associates (171, 172, 173, 174) have studied extensively such methods of selective absorption. These authors use specially activated magnesium hydroxide and carbonate, and shake a solution of the impure carotene with a given amount of the absorbent. These same authors have developed an absorbent "X" which absorbs xanthophyll but not carotene, and an absorbent "L" which removes considerable impurities from extracts of watermelon, ripe tomatoes, *etc.*

Peterson (143) has criticized these methods on various grounds, *e.g.*, (1) special techniques are required for preparing the absorbents, (2) the proper activation of the absorbent must be frequently checked in order to determine whether it is absorbing β-carotene instead of impurities. Further this technique does not provide a means of determining the number of pigments present or for studying their spectral properties.

Soda ash (175), magnesia (173, 174, 176, 177), and dicalcium phosphate (166, 178), have been recently suggested for the separation of the other fat-soluble pigments from the carotenes. In all these separations, petroleum ether was used as the solvent, with one exception; Strain (176) used dichloroethylene.

Strain's method (177) consists of the extraction of 1 kg. of dried powdered carrot leaves with 5.5 l. of dichloroethane and the extract is then reduced in volume to 100 cc. *in vacuo.* This concentrate is then passed through a column (6.6 × 22 cm.) of Micron Brand magnesium oxide diluted 1:1 with Hyflo Super Cel. All of the carotene appeared in the eluate when the column was washed with fresh dichloroethanol. A modification of this method, for use with small quantities of leaf material, consists in putting the powdered dried leaf material (1 g.) on top of the absorbent in the column and percolating it with the dichloroethane, thus extracting and separating the carotene in one operation.

Soda ash was introduced by Kernohan (95), and Strain points out (97) that, under the conditions employed, the carotene is not absorbed, so that there is little or no decomposition and the carotene in the eluate is not contaminated with colored artefacts from the other leaf pigments.

Kernohan's method consists of extracting a 1 g. sample of alfalfa in a stoppered flask overnight with 100 ml. of petroleum ether. The petroleum ether extract is then drawn through a column of finely-divided soda ash by suction, and the column washed with fresh petroleum ether. A chromatogram of the various petroleum ether-soluble plant pigments develops on the column, and the carotene passes through into the filtrate. Magnesium oxide has been extensively used in the chromatographic analysis of petroleum ether extracts (179). This absorbent separates cryptoxanthol, neo-cryptoxanthol, β-carotene, K-carotene, α-carotene, and other unidentified pigments which are biologically inactive. Gillam *et al.* (180), and Beadle & Zscheile (22), have reported that magnesium oxide will not separate β-carotene and neo-β-carotene.

Moore (166) has pointed out that, in the phasic separation of the carotenes from feces or from plant material that has been stored as hay or made into silage, certain non-carotene chromogens appear in the petroleum ether phase. This failure of the Willstätter-Stoll phasic separation had been indicated before (181, 182). It was shown in 1939 (152), that these non-carotene chromogens could be removed by extracting the petroleum ether phase with aqueous diacetone alcohol.

Moore (166) found that a chromatogram carried out with dicalcium phosphate caused no destruction of carotene and held the non-carotene pigments in the upper part of the Tswett column when petroleum ether was used as the solvent.

Mann (165) has used bone meal, previously extracted with a mixture of petroleum ether, acetone, and ether, for the separation of β-carotene and neo-β-carotene. Mann has also used this adsorbent for the separation of vitamin A from xanthophylls in the presence of egg yolk sterols.

The most useful absorbent for the separation of β-carotene and the neo-β-carotenes is calcium hydroxide (159). In the hands of Kemmerer and Fraps (161), this absorbent has been used to separate neo-β-carotene B, β-carotene and neo-β-carotene U.

After phasic separation, the petroleum ether extracts of most plant materials show five main bands in their chromatograms on calcium hydroxide. These are, reading from the top to the bottom of the chromatogram:

(1) *Impurity A:* This band may consist of several zones of yellow, red, or bronwish-yellow pigments.

(2) *Neo-β-carotene U:* Light orange pigment just above the β-carotene zone. This pigment separates late in the analysis.

(3) *β-Carotene:* Wide reddish-orange zone.

(4) *Neo-β-carotene B:* Yellow-orange band immediately below the β-carotene band.

(5) *α-Carotene:* Orange band that is not present in most materials in appreciable amounts.

In some materials a small band is occasionally found just below this α-carotene band. This band is usually isolated along with the α-carotene. Occasionally, it will be found that clean separation of the bands does not occur; in such cases, the chromatogram is developed by washing with petroleum ether containing a small amount of acetone (1-2%).

D. THE DETERMINATION OF CAROTENE

It has been pointed out above that unless certain chromatographic methods are employed, the final carotene solution may give an abnormal absorption spectra, due to the interference of the neo-β-carotene and other chromogens.

The usual method for the determination of the concentration of crude carotene is to determine the optical density of the solution at 450 mμ in petroleum ether or 463 mμ in $CHCl_3$, and then compare the $E^{1\%}_{1\,\text{cm.}}$ calculated from this value with that of a standard pure sample of β-carotene in the same solvent. Knowing the concentration of β-carotene in the standard, the concentration in the unknown is calculated by simple proportion.

The specific absorption coefficients of β-carotene vary somewhat in different solvents. The following table (XIV) lists the specific absorption coefficients for the more common solvents.

TABLE XIV

Solvent	Ref.	Wave length mμ. 436.0	450.0	460.0	478.0
Hexane	(22)	196	258	206	228
Hexane	(175)	198	259	210	231
Redistilled Skellysolve B	(175)	193	253	203	224
5% Acetone in redistilled Skellysolve B	(175)	193	253	205	224
20% Ether in EtOH	(183)		249[a]		
Carbon disulfide	(157)		243[a]		
Chloroform	(184)		220[a]		
Ether	(185)		208[a]		

[a] The values at the highest maximum.

The following precise spectral characteristics of various carotenes in hexane have been collected from the literature (Table XV).

TABLE XV

Carotenoid	Ref.	Maxima mμ	$E^{0.1\%}_{1\ cm.}$
α-Carotene	(186)	474	250
		445–6	272
		422	179
		333–4	14.9
		267	49.3
β-Carotene	(186)	478	228
		452	260
		272	40.6
Neo-β-carotene B	(22)	467	166
		462.5	164
		442.5	206
Neo-β-carotene U	(186)	474	218
		463	196
		446–7	252
		342–3	23.3
		265	32.8
δ-Carotene	(187)	493	242.5
		461.2	272
		436	188
Cryptoxanthol	(188)	415.5	246.3
		478	216.5

E. METHODS OF PROVITAMIN A ASSAY

(*1*) *The Method of the A.O.A.C.* (*189*)

Reagents.

(*a*). *Alcoholic Potash Soln.* Dissolve 12 g. of KOH in 100 ml. of alcohol.

(*b*) *Petroleum Benzine.* Use petroleum benzine or Skellysolve F, b. p. 30-70° C.

(*c*) *Methanol.* 90%. Place 100 ml. of H_2O in 1 liter volumetric flask and dilute to volume with absolute methanol.

(*d*) *Standard Carotene Soln.* Purify commercial crystalline carotene as follows: Dissolve 0.1 g. in 2 or 3 ml. of $CHCl_3$, precipitate with 25 ml. of methanol, filter, wash once with methanol, and dry in vacuum desiccator for not more than 1 hr. Dissolve 20 mg. of the purified carotene in 1-2 ml. of $CHCl_3$ and dilute to 100 ml. with petroleum benzine.

(*e*) *Activated Magnesium Carbonate.* Place 2.5 g. of U.S.P. light $MgCO_3$ in 2 oz. bottle. Add 50 ml. of the purified carotene soln., stopper tightly, shake vigorously 1 min., and centrifuge without removing stopper. Determine carotene. If no carotene is lost, the $MgCO_3$ is suitable for use; if more than 1% carotene is lost, thoroughly mix 50 g. of the $MgCO_3$ with 10 ml. of H_2O. Test mixture with the carotene soln. If the carotene is still removed, add 5 ml. more of H_2O and test again. Add H_2O in 5 ml. portions until the $MgCO_3$ no longer adsorbs carotene.

(*f*) *Calcium Hydroxide.* $Ca(OH)_2$ or commercial hydrated lime low in Mg. Sift through 48-65 mesh sieve.

(*g*) *Potassium Dichromate Soln.* 0.02%. Weigh accurately 0.2000 g. of $K_2Cr_2O_7$, dissolve in *ca.* 100 ml. of H_2O, and dilute to 1000 ml.

(*h*) *Potassium Dichromate Soln.* 0.10%.

I. Phasic Separation

Preparation of Solution.

(*a*) *Hays and Dried Plants.* Weigh out 1-5 g. of sample, transfer to 200 ml. Erlenmeyer flask, and for each gram of sample add 20 ml. of the freshly prepared KOH soln. Fit flask with reflux condenser and boil contents on steam bath or hot plate 30 min. If portions of sample collect on sides of flask, wash down with alcohol from wash bottle. Cool contents of flask, (volume of petroleum benzine to be used later for extraction may be reduced by filtering directly, after cooling, through sintered-glass funnel of No. 3 porosity, extracting residue with small portions of petroleum benzine until solvent is colorless, and proceeding as directed below). Add 100 ml. of petroleum benzine and after shaking for 1-2 min. and allowing sediment to settle, decant mixture into 500 ml. separator. Repeat procedure twice more with 25 ml. portions of the solvent, breaking up residue, which sometimes forms an adherent mass, by shaking with 10-15 ml. of alcohol. After two or three additional extractions with 20 ml. portions of solvent (soln. usually comes off colorless), discard residue.

Remove xanthophyll with 25 ml. portions of 90% methanol, shaking 2 min. each time. Continue these extractions until methanol is colorless (6-12 washings, depending on amount of xanthophyll in sample). Wash the petroleum benzine containing the carotene twice with 50 ml. of H_2O to remove the methanol, concentrate under reduced pressure if necessary, filter into volumetric flask (of such size as to obtain convenient concentration for measurement of carotene) through filter paper upon which is placed a small amount of anhydrous Na_2SO_4, and adjust to volume.

This carotene soln. may be further purified by shaking a 50 ml. aliquot with 2.5 g. of the activated $MgCO_3$ in a 2 oz. tightly stoppered widemouth bottle for 1 min., and centrifuging.

(*b*) *Fresh Green Materials.* For samples to be analyzed soon after gathering (1-2 hr.), place 100 g. of material in a large evaporating dish, soak in 100 ml. of alcohol 5 min., and cut up with scissors. Add 100 g. of clean white sand (free or organic matter), and grind until a uniform mixture is obtained. For samples necessitating shipment or delay in analysis, place 100 g. of material in a tared fruit jar with a weighed quantity of alcohol or methanol. Seal jar with rubber under lid. Upon arrival at laboratory, weigh jar and contents, and, to obtain weight of sample, subtract weight of jar and alcohol from total weight. Pour contents of jar into evaporating dish, cut up with scissors, and grind with sand as directed above.

Decant liquid from solid part through cheese cloth and make to volume. Weigh solids. Take aliquots of both solid and liquid, equivalent to 5 g. of fresh untreated material, and mix together for carotene analysis. Saponify by boiling 30 min. in 50 ml. of the KOH soln. Cool, add 50 ml. of petroleum benzine, and decant liquid into separator. Transfer residue to mortar and grind with pestle, first with 15 ml. portions of petroleum benzine, and then with mixture of 5 ml. of alcohol and 15 ml. of petroleum benzine, until no further color is extracted. Decant washings into the separator and proceed as directed in (*a*) beginning "Remove xanthophyll."

If crude carotene on dry basis only is desired, analyze preserved samples by pouring off as much of the alcohol as possible, grinding solid portion in food chopper, weighing out 5 g. and proceeding as directed in (*a*), determining dry matter on separate portion.

(*c*) *Fresh Sweet Potatoes.* Proceed as directed in (*a*), except to substitute alcohol for the alcoholic KOH soln.

(*d*) *Fresh Carrots, Apricots and Similar Materials.* To 5 g. of finely-ground sample, add 40-50 ml. of alcohol and reflux 15 min. Filter through fluted filter or fritted-glass funnel and wash residue with small portions of alcohol. Remove residue, add 25 ml. of alcohol, and again reflux for 15 min. Filter and wash as above. If residue contains more than very slight amount of yellow color, reflux a third time. To combined alcoholic solns. add *ca.* 25 ml. of the KOH soln., and reflux *ca.* 5 min. Add 50-100 ml. of petroleum benzine. Shake, and then add 100-150 ml. of H_2O and allow layers to separate. Draw off aqueous alcoholic layer and re-extract with 25-50 ml. of petroleum benzine. Repeat this extraction until petroleum benzine layer separates colorless, or only slightly colored. Proceed as directed in (*a*), beginning "Remove xanthophyll."

(*e*) *Dehydrated Sweet Potatoes, Carrots, Apricots, and Similar Materials.* To 1 g. of finely-ground sample add *ca.* 4 ml. of H_2O and allow mixture to stand 30-40 min. Add 40-50 ml. of alcohol and proceed as directed in (*d*).

(*f*) *Dehydrated Green Leafy Vegetables.* Proceed as directed in (*a*).

(*g*) *Canned Foods.* Pour contents of can onto piece of cheesecloth and allow liquid to drain. Grind solids in food chopper and proceed as directed in (*a*).

(*h*) *Butter and Other Fats, Egg Yolks, and Liver.* Weigh 5 g. of sample and proceed as directed in (*a*).

(*i*) *Blood Plasma.* Weigh 10-30 g., reflux 15 min. with 25-50 ml. of the KOH soln., and proceed as directed in (*a*).

(*j*) *High Lycopene-Containing Materials (Tomatoes and Watermelons).* Place 1-2 g. of the activated $MgCO_3$ in a glass tube 5-8 mm. wide and *ca.* 15 mm. tall, constricted at one end and plugged with wad of cotton. Apply suction and pack the $MgCO_3$ firmly but not too tightly with cork having smooth surface, attached to glass rod.

Weigh 5 g. of sample and proceed as directed in (*a*). Dilute the final petroleum benzine soln. of carotene to 200 ml., evaporate 50 ml. aliquot *in vacuo* to 10 ml., and run through the $MgCO_3$ column. Wash column with petroleum benzine until all carotene is washed through. (Red band of lycopene will pass slowly down column; be careful not to wash it through the column.) Dilute soln. that passes through column to appropriate volume.

II. Chromatographic Separation

Extraction.

In the extraction of pigments, avoid heating because it produces neo-β-carotene. For some products the phasic separation with methanol can be omitted.

(*a*) *Fresh Green Materials, Butter, Egg Yolk and Fresh or Dried Fecal Material.* Chop fresh material into pieces *ca.* ¼″ long and weigh 10 g. into chamber of Waring Blendor or similar apparatus. Add 150-200 ml. of the KOH soln. and grind 5 min. Proceed as directed in *I*(*a*), beginning "Add 100 ml. petroleum benzine." Instead of determining carotene directly, treat the final petroleum benzine soln. as directed [under adsorption].

(*b*) *Dried Hay, Grass, and Dehydrated Leafy Vegetables.* To 5-25 g. of sample, depending on carotene content, add 150-200 ml. of the KOH soln., and let stand overnight in refrigerator. Then grind in Waring Blendor or similar apparatus and proceed as directed in (*a*).

(*c*) *Fresh Sweet Potatoes.* Proceed as directed in (*a*), except to use alcohol instead of the KOH soln.

(*d*) *Fresh Carrots, Apricots, and Similar Materials.* Extract with alcohol as directed in (*c*). To the alcoholic extract add 25 ml. of the KOH soln., and let stand 5 min., and then extract with petroleum benzine, *etc.*, as directed in (*a*).

(*e*) *Dehydrated Fruits and Vegetables High in Sugar, Such as Dried Apricots, Carrots or Sweet Potatoes.* Weigh 5-10 g. of sample, add 50-100 ml. of H_2O, and allow to stand overnight in refrigerator. Then proceed as directed in (*a*).

Adsorption.

In constricted end of glass tube, 5-8 mm. wide and 15-20 cm. high, place small wad of cotton. Apply suction and, by means of smooth-surfaced cork attached to glass rod, pack the $Ca(OH)_2$, 0.2 g. at a time, into the tube gently but firmly until column *ca.* 10 cm. long is obtained.

Place 2-5 ml. of petroleum benzine in column and apply suction. Before all the petroleum benzine has been drawn into column, add the petroleum benzine soln. of carotene from *II*(*a*). When this has been nearly drawn into column, add petroleum benzine. (Always keep surface of column covered with petroleum benzine.) Wash column with petroleum benzine until zones of pigments have separated (2-4 hr.). Scrape each zone from column into beakers, using wire flattened at one end. Keep powdered material in beakers covered with petroleum benzine. Extract each pigment from powdered material by stirring with petroleum benzine containing 2% alcohol. If $Ca(OH)_2$ does not settle readily, add alcohol dropwise until it coagulates. Decant liquid into separator and extract the $Ca(OH)_2$ again. Repeat extraction until all pigment is removed. Combine extracts and wash 3 times with H_2O, to remove alcohol. If extracts are still cloudy, wash once with HCl (1 : 100) and then once with H_2O. Dry over anhydrous Na_2SO_4, dilute to suitable volume in graduated flasks, and determine amount of each pigment (expressed as carotene) by the method given below, or by a colorimetric method (30).

With most materials, zones of pigments found in column, starting from top, are as follows:

(1) *Impurity A.* May consist of several zones of yellow, red, or brownish-yellow pigments.

(2) *Neo-β-carotene U.* Light orange pigment just above β-carotene zone. This pigment separates late in the analysis.

(3) *β-Carotene.* Wide reddish-orange zone.

(4) *Neo-β-carotene B.* Yellow-orange zone immediately below β-carotene zone.

(5) *α-Carotene.* Orange zone that is not present in most materials in appreciable amounts.

Another small zone is sometimes present below the α-carotene zone. For all practical purposes, it may be removed with the α-carotene. If zones do not separate, wash the column with petroleum benzine containing 1-5% of acetone.

Determination.

(*a*) *Spectrophotometric Method.* For each determination make optical density measurements at wavelengths of 4500, 4700 and 4800 Å. Using the following specific absorption coefficients calculated for β-carotene at these wavelengths, determine carotene concentration for each wavelength, take average, and report results to 0.1 p.p.m.

SPECIFIC ABSORPTION COEFFICIENTS

(Absorption of 1 g./liter in 1 cm. cell)

Wavelength, Å.	Skellysolve B.P. 60-70	Petroleum Benzine B.P. 40-60°
4500	238	243
4550		231
4700	200	207
4800	212	212

Modifications and improvements of this method have been described by Kemmerer (189a, 189b).

(2) The Dicalcium Phosphate Method (166, 178)

To activate dicalcium phosphate, Moore (178) used the following procedure:

One hundred g. of the inactive dicalcium phosphate (Merck's reagent) are weighed out and placed in a 2 l. Erlenmeyer flask. A quantity greater that 100 g. usually causes considerable bumping. Five g. of KOH are added in 1 l. of water. The contents of the flask are boiled on a hot plate for 15-30 min., transferred to a Buchner funnel and washed with 0.5-1 l. of water. The phosphate is placed in an evaporating dish and dried for 24 hr. at 100° C. and is then broken up in a mortar, after which it is ready for use.

Moore's method (166) of separating the carotene from other pigments for estimation of the former is as follows:

Two to 4 g. of plant material, depending on the carotene content, are used. Dried materials are finely ground to get effective extraction, silage is ground through a food chopper, and fresh material is cut up with scissors. After weighing the sample, the silage or fresh material is quickly ground in a mortar with quartz sand.

The material is transferred to a 250 ml. Erlenmeyer flask and 50 ml. of 95% aldehyde-free ethyl alcohol are added. In the case of the silage and fresh material, the alcohol is used in the grinding process and transfer.

The sample is then refluxed for 30 min. on a water bath and cooled, the alcohol extract is decanted into a 500 ml. separatory funnel, and 25 ml. of alcohol are added to the residue; it is then refluxed for another 30 min. period, and the second extract is decanted into the first. The residue is further extracted with petroleum ether and sufficient alcohol is added to keep the residue dispersed. Twenty-five ml. of alcohol are generally used in this process, making a total of 100 ml., and about 50 ml. of petroleum ether. Sufficient water is then added to the extract to make the alcohol concentration 80%.

The lower layer is separated from the upper layer, after which it is extracted 3 times more with 30 ml. portions of petroleum ether. This does not extract all the color from the alcohol phase, but, as practically all the carotene is removed by the first two extractions, there is no need for further extraction.

All the alcohol must be removed from the petroleum ether extract, otherwise all the other pigments will pass through the adsorbent with the carotene. For this reason, the petroleum ether extract is then washed 6 or 7 times with 100 ml. portions of tap water, and concentrated to about 20-25 ml.

The tubes used for the dicalcium phosphate column can easily be made from ordinary test tubes approximately 19 cm. long and 2.2 cm. in diameter, by blowing out the bottom and attaching a small piece of glass tubing at the end. The glass tube is supported by a 250-ml. Erlenmeyer flask, held by a clamp to catch the filtrate. A small piece of cotton is used to plug the small opening in the lower end of the tube, and a larger piece is placed on top. The tube is filled a little over half full with dicalcium phosphate, which is packed lightly by application of vacuum from an ordinary water pump and by use of a small plunger made with a glass rod and a cork of the proper size. A small amount of anhydrous sodium sulfate is placed on top of the phosphate, so that the tube is about half-filled by the column.

Thirty ml. of petroleum ether are run into the column, followed by the extract, (concentrated to 20-25 ml.) and then 60 more ml. of petroleum ether. The various pigments are adsorbed in bands, while the carotene is washed on through the column. The carotene filtrate is then concentrated, if necessary, and made up to volume, and the carotene concentration is determined.

(3) The Magnesium Oxide Method

Silker *et al.* (175) have used a mixture of Skellysolve B and acetone for extracting the carotenoids from alfalfa. The β-carotene is separated from the other pigments chromatographically on magnesium oxide diluted with Hyflo Super Cel and then determined along with neo-β-carotene B spectrophotometrically. The method follows:

Extraction of Carotene. One or 2 g. of finely-ground alfalfa (preferably passing through a 40-mesh screen) are weighed into an Erlenmeyer flask or sample bottle and covered with 60 ml. of a mixture of 1 part acetone to 2 parts Skellysolve B. The mixture is shaken, and the tightly stoppered container then set aside in the dark for 16-18 hr., usually overnight. The extract is filtered through a Buchner funnel and the residue thoroughly washed, by decantation, with several small portions of Skellysolve, followed by heating on a steam cone to drive off most of the remaining acetone and to concentrate the solution to a volume of approximately 40 ml. (15 min. heating is usually sufficient).

Separation of Carotene from Other Pigments. A chromatographic separation of carotene from other pigments is made on a column of 2 parts Hyflo Super-Cel and 1 part magnesia (Micron brand No. 2541). The columns are made as described by Wall and Kelley (190) but are shorter in length (8-10 cm.). After adsorption of the pigments, the carotene is eluted with a 4% acetone-Skellysolve B mixture.

Estimation of Carotene. The solution of carotene is made up to volume and analyzed with a Beckman spectrophotometer for β-carotene and neo-β-carotene B, using the wavelengths and absorption coefficients suggested by Beadle and Zscheile ((22), see below).

(4) The Calcium Hydroxide Method (191, 161)

A quantity of the plant material to yield 100-200 γ of crude carotene is agitated in a Waring Blendor with 150 ml. of 12% alcoholic KOH for 5 min. at room temperature. Skellysolve F is then added, and the mixture diluted with water, and the crude carotene extracted with Skellysolve. The extract is dried with sodium sulfate and made up to volume, and the crude carotene is determined by means of a photoelectric colorimeter. The solution is concentrated to 10-15 ml. and the constituent pigments separated chromatographically on a column of calcium hydroxide (passing 65 mesh). The chromatographic analysis is carried out in a tube 5.8 mm. wide and 15-20 cm. high constricted at one end, the column being cooled by a jacket through which ice-water passes. The narrow end of the adsorption tube is plugged with cotton and the calcium hydroxide is put in, in 0.2 g. portions, and tamped down gently while suction is applied. Portions of the calcium hydroxide are added until the column is about 10 cm. high. The petroleum ether solution of the crude carotene is put into the column and the chromatogram developed with fresh petroleum ether until the bands are separated. The separated bands are then scraped mechanically from the column into beakers and always kept covered with petroleum ether. The pigments are eluted from the calcium hydroxide by means of petroleum ether containing ethanol. The eluates are made up to volume and concentration of the pigments determined.

(5) The Bone Meal Method (165)

The following method for the carotenoids is due to Mann (165).

Weigh a suitable quantity of dried grass (3 g. of a sample of poor quality or 1 g. of one of good quality) and grind with fine, hard sand with an agate pestle in a porcelain or agate mortar, until the grass is smooth and flourlike. If the sample consists of fresh leaf material and silage, grind it with anhydrous sodium sulphate together with the sand, and, if it is silage, neutralize the acidity by further addition of 1 g. of anhydrous sodium carbonate for each g. of silage. Next transfer the sample and sand to a Soxhlet thimble in a straight-through drip-type extraction tube, fitted with a 100-ml. round flask and a reflux condenser, rinse the mortar until free from color with a few ml. of ether into the flask, and extract on a water bath with a mixture (1 : 3) of acetone and peroxide-free ether, freshly distilled from sodium. Maintain the water bath at a temperature just sufficient to boil the acetone-ether mixture. (If β-carotene determination only is required, use a mixture of light petroleum and acetone (3 : 1) for extraction and for rinsing the mortar.) After 30 min. remove the thimble, re-pound the grass and re-extract for 15 min. Again re-pound and re-extract for a further 15 min. Any color then remaining in the grass will be due to chlorophylls.

Transfer the acetone and ether extract of the grass (or petroleum-acetone extract if only β-carotene is being determined) to a 300-ml. round-bottomed flask and distil on a water bath until only *ca.* 5 ml. remain. Complete the evaporation by blowing nitrogen or carbon dioxide into the flask, which is meanwhile rotated on the bath. Then add 10 ml. of light petroleum and blow into the flask as before until the extract has a viscous consistence. Next add 30 ml. of light petroleum, mix the contents of the flask by shaking, and immediately cool under running water.

Preparation of Adsorption Column. Bone meal, (commercial steamed bone flour, feeding bone flour) previously extracted for 24 hr. in a straight-through drip-type extraction tube with a mixture of light petroleum (b.p. 40°-60° C.), acetone and ether (3 : 1 : 1 to remove fat and sterols) until no more color can be extracted, is used as adsorbent for chlorophylls, neo-β-carotene and xanthophyll as follows:

Mount a glass tube (6 in. long by 1 in. internal diam., and having a narrower open stem 3 in. long at the base) on a filter-flask, put a small plug of cotton wool in the bottom of the tube and press it firmly, so that *ca.* ⅜ in. of the stem is filled with wool, leaving ¼ in. in depth in the tube proper. Next place the extracted bone meal in the tube to a depth of 2 in., then another layer of cotton wool, and finally a ¼ in. layer of anhydrous sodium sulphate. Lightly tap the tube during filling, and press the contents gently, but not too tightly, with a flat-ended rod. To obtain bright solns. of β-carotene pass *ca.* 30 ml. of acetone through the column of bone meal, and then remove the acetone from the column with about 50 ml. of light petroleum.

Separation of β-Carotene from Other Pigments. Apply gentle suction to the column, and pass some light petroleum through it. Before the column is dry add the grass extract in light petroleum and rinse the flask with light petroleum until no petrol-soluble pigment remains, the successive washings being added to the column. If xanthophyll and neo-β-carotene determinations are required (*cf.* next paragraph) add a few ml. of ether to the flask to dissolve any xanthophyllic pigment adhering to the flask wall. Then close the flask with a cork and set it aside until needed. Elute the column with light petroleum until no further color can be removed, and throughout the separation maintain it in a flooded condition. Chlorophylls, neo-β-carotene and xanthophyll will remain adsorbed, and the eluate will contain the total β-carotene; 100 ml. of light petroleum are usually sufficient to elute the whole of the β-carotene from any of the suggested samples. Finally estimate the β-carotene in the yellow soln. by the usual spectroscopic or colorimetric methods.

Separation of Chlorophylls, Neo-β-Carotene and Xanthophyll. For the separation of chlorophylls, neo-β-carotene and xanthophyll, the following procedure has been adopted. Elute these pigments, adsorbed on the bone meal, by passing ether through the column; it is impossible to elute the whole of the chlorophyll with ether, but in practice 100 or 150 ml. are sufficient to remove the yellow pigments and most of the chlorophylls. Return the eluate to the *original* round-bottomed flask and reduce to *ca.* 5 ml. by distillation from a water bath at as low a temperature as possible. Add 5 ml. of color-free 30% methyl alcoholic potash (freshly prepared) and heat the extract under reflux with an air condenser for 10 min. with periodical shaking.

Cool the flask and transfer the contents to a 500 ml. separatory funnel, using *ca.* 50 ml. of peroxide-free ether in all to rinse the inside of the flask, adding the rinsings to the funnel. Also use 60 ml. of water, in portions, to rinse the inside of the flask, add these to the contents of the funnel, and shake the whole *gently*. After standing, the yellow pigments will be found dissolved in the upper ether phase while the potassium chlorophyllins remain with the lower aqueous phase. Drain the latter phase into another separating funnel, and re-extract it with further 50 ml. portions of ether, until no more color is extracted. After 2 or 3 extractions, the shaking may be more vigorous. The extraction of a small quantity of chlorophyllin along with the yellow pigments will not vitiate subsequent operations.

Combine the ethereal extracts, wash with water until the washings give no color with phenolphthalein, evaporate by distillation, and blow nitrogen or carbon dioxide into the flask, exactly as described for the preparation of the original extract; take up the residue in *ca.* 30 ml. of light petroleum, pass the soln. over a fresh column of bone meal, prepared as previously described, and then pass *ca.* 150 ml. of light petroleum through the column so that neo-β-carotene and xanthophyll may be adsorbed. Add a few ml. of acetone to dissolve any adherent xanthophyll, cork the flask and set it aside. After adsorption of these pigments, the column, while still damp with light petroleum, is transferred immediately to another filter-flask and flooded with benzene (thiophene-free), gentle suction being applied.

Next elute the column with benzene until the eluate runs colorless. The eluate contains the whole of the neo-β-carotene; $E^{1\%}_{1\,\mathrm{cm.}}$ 454 mμ (benzene) 1900 (provisional). Remove the benzene remaining on the column with light petroleum, elute the xanthophyll with acetone, and add the eluate to any xanthophyll in the flask which has been corked and set aside; $E^{1\%}_{1\,\text{cm.}}$ 447 mμ (acetone) 2400 (provisional).

Lastly, remove the acetone with light petroleum, leaving the column ready for many further similar separations.

(6) The Differential Spectroscopic Method

a. Plant Materials. Beadle and Zscheile (22) have developed a purely spectroscopic method for the determination of the components of the "homogeneous" β-carotene fraction. These authors isolate the epiphasic pigments by the following method:

Ten g. of fresh spinach leaves were extracted (5 min.) with 125 ml. of cold (20°) diacetone alcohol in a Waring Blendor, and the mixture filtered through paper on a Jena slit sieve filter. The percentage of water in the filtrate was 6.5 by volume. The carotenes were transferred to hexane by four extractions (25 ml. each) in a 500 ml. separatory funnel. The proper concentration of water is important for this transfer. The small amount of carotenols and chlorophylls in the hexane was removed by three extractions (25 ml. each) with aqueous diacetone alcohol (100 parts diacetone alcohol plus 7 parts of water by volume) and the last traces of chlorophylls removed by shaking for 1 min. with 25 ml. of 20% KOH in methanol at room temperature. The final hexane solution of carotene was washed three times with water and dried over sodium sulphate. The absorption measurements were made at 25 Å intervals from 3800 to 5000 Å.

Recovery experiments carried out with added β-carotene in the above purification showed that there was very little loss of total pigment.

The absorption curve obtained by Beadle and Zscheile for this extract is shown in Fig. 6 and compared with those of β-carotene and neo-β-carotene.

It will be seen that the curve of the "carotene" fraction from spinach is low toward the red end of the spectrum and high toward the blue, as compared with the curve for pure β-carotene.

The total concentration (c) of β-carotene and neo-β-carotene can be obtained from the absorption value at a coincident point (4360 Å) of the absorption curves (Fig. 6) of the two substances by means of the following equation:

$$C = \frac{\log_{10} \frac{I_0}{I}\,(4360)}{196}$$

The percentage of the second component is given by the equation:

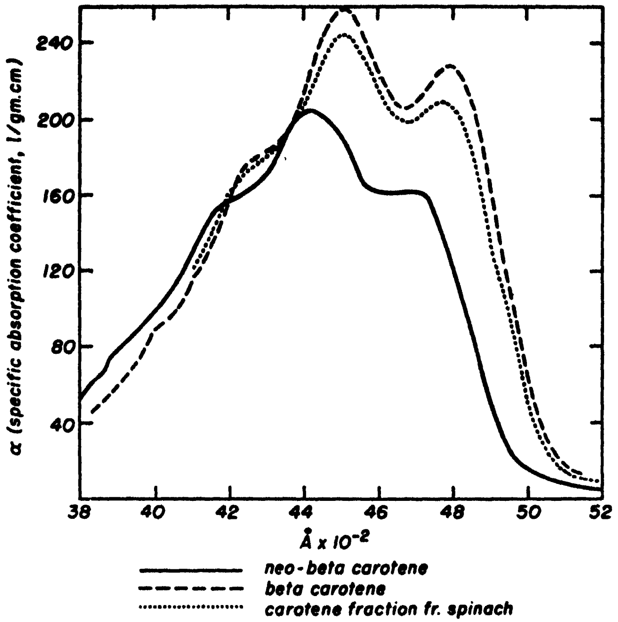

Fig. 6. Absorption spectra in hexane solution of β-carotene, neo-β-carotene and carotene fraction from spinach. (after Beadle & Zscheile (22)).

$$\% \text{ component } 2 = \frac{\dfrac{\log \dfrac{I_o}{I}(4780)}{C} - 132}{96} \times 100$$

The reader is referred to Zscheile's papers (22, 192) for the complete derivation of these equations. These authors (22) give the following example of the use of these equations for an extract of spinach prepared by the method given above:

$$\log \frac{I_o}{I}(4360) = 0.341;\ \log \frac{I_o}{I}(4780) = 0.367$$

$$c = \frac{0.341}{196} = 0.00174 \text{ g./l.}$$

$$\% \text{ Component } 2 = \frac{\dfrac{0.367}{0.00174} - 132}{96} \times 100 = 82.3$$

In the above Component 1 = neo-β-carotene and Component 2 = β-carotene.

The analytical constants used by Beadle and Zscheile (22) for the differential spectroscopic method of assay of β-carotene and neo-β-carotene are given in Table XVI.

TABLE XVI

Wave-length A	Specific absorption coefficients in hexane	
	β-Carotene	Neo-β-carotene
4850	200	81.3
4780	228	132
4360	196	196

b. Butter. Zscheile and his coworkers (154) have shown that the absorption spectra of β-carotene and neo-β-carotene in diethyl ether solution showed significant quantitative differences in both wavelength and intensity of absorption from those given by the same pigments in hexane solution (22). These authors have suggested that, since the curves of these two pigments are coincident at 437 mμ. in diethyl ether, this wavelength should be chosen for the determination of total carotenoids. In hexane solution, the curves are coincidental at 436 mμ.

The procedure of Zscheile *et al.* for the preparation of the extract of butter for the assay of carotene and vitamin A is as follows: (154)

Weigh a 10 g. sample of filtered butterfat (liquid or solid) or of crude butter (solid) into a 300 ml. saponification flask. Add a mixture of 5 ml. of saturated aqueous KOH and 20 ml. of aldehyde-free methanol. Connect a reflux condenser with a ground-glass joint to the flask and boil the mixture on a hot plate for 10 min. Dilute immediately with 40 ml. of distilled water and cool to room temperature under the tap.

Transfer to a separatory funnel, using 50 ml. of additional water to rinse the flask. Add 100 ml. of peroxide-free diethyl ether to extract the non-saponifiable fraction. The ether should give a negative Jorissen test (193). After separation of the two phases, extract the aqueous layer three more times, each time with a 50 ml. portion of ether. Combine the ether extracts and wash with distilled water until free of alkali (4-6 times). Reduce the volume of ether to about 80 ml. on a steam bath, recovering the ether with a condenser. Dry over anhydrous sodium sulfate and make to a volume of 100 ml. Place in refrigerator if spectroscopic observations cannot be made at once.

Make spectroscopic observations the same day if possible; this is especially desirable for the ultraviolet readings. Use wavelengths 3240 and 4370 Å for vitamin A and total carotenoids, respectively. Be certain to employ for the solvent (I_o) cell, ether that came from the same bottle as that used for the preparation of the extract. This is important to avoid errors due to absorption in the ultraviolet by solvent impurities.

Methanol of a good grade must be used and an occasional blank determination is advisable to avoid spurious results due to impurities from the methanol.

The ether solution is then divided into two parts, one for the determination of vitamin A by means of the Carr-Price antimony trichloride method (194). The ether is evaporated carefully to dryness and the residue is then dissolved in Skellysolve B and extracted repeatedly with 92% aqueous methanol or 94% aqueous diacetone alcohol to remove the xanthophylls. The petroleum ether is then dried with anhydrous sodium sulfate and the carotenes determined by measuring the transmission in the 440 mμ region with a photoelectric filter photometer.

Zscheile *et al.* (168) have studied the effect of clarification, adsorption, acid extraction, and freezing upon the characteristic absorption curves obtained from the carotenoids of butterfat. They recommend that samples should be clarified before analysis since the materials removed may not be uniform in all samples, and further such a procedure places the assay on a fat basis thus eliminating differences in water and salt content.

4. VITAMIN D

The spectroscopic method for the assay of vitamin D (195, 196) suffers from the same limitations as the spectroscopic determinations of other natural products, *e.g.*, the absence of substances which absorb in the same region of the ultraviolet spectrum as the D vitamin is essential.

All the D vitamins (D_2, D_3 and D_4) have almost identical spectra showing a characteristic single broad band with a maximum at *ca.* 265 mμ. The extinction coefficients show slight variations being highest for vitamin D_3 ($E^{1\%}_{1\text{ cm.}} = 500$) and lowest for D_2 ($E^{1\%}_{1\text{ cm.}} = 460$) ; D_8 has $E^{1\%}_{1\text{ cm.}} = 470$ (197).

Thus, it is impossible to determine the amounts of each individual vitamin D in a mixture and, furthermore, in fish oils, the vitamin A present, absorbing at 328 mμ with a $E^{1\%}_{1\text{ cm.}}$ four times as great as that of the vitamin D's, completely overshadows that of the latter.

Methods have been suggested (199) for separation of vitamins A and D by chromatographic means on aluminum oxide (200), *etc.*, after saponification, but the losses incurred in such processing make the accuracy of the method very questionable.

The spectroscopic method can only be applied to almost pure crystalline vitamins D by the comparison of the $D^{1\%}_{1\text{ cm.}}$ at 265 mμ of the unknown with that of the pure vitamin D at the same wavelength and with the same solvent (hexane, ether, *etc.*).

The more reliable non-biological methods for the estimation of the D vitamins depend on the measurement of the color produced with antimony trichloride and are reviewed by Ewing *et al.* (201). These authors describe the chromatographic separation of vitamin D from adsorbing impurities.

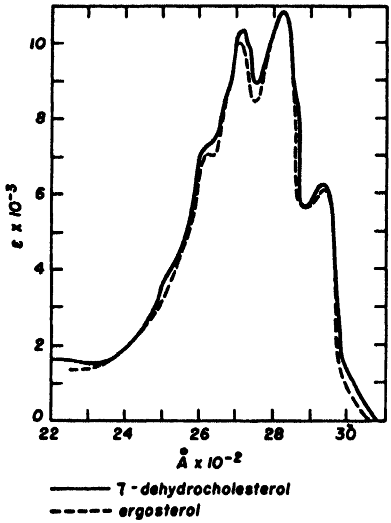

Fig. 7. Absorption curves of 7-dehydrocholesterol and ergosterol in EtOH (after Hogness *et al.* (198)).

5. VITAMIN E

The determination of the vitamins E by physical means suffers from several drawbacks, not the least of which is the impossibility of differentiating between the α-, β- and γ-isomers, which have different biological potencies.

Many substances occurring in natural sources of vitamin E and in tissues show ultraviolet absorption in the same region of the spectrum as the vitamin. Furthermore, the tocopherols occur, in part at least, as their esters which show a shift in the maximum to *ca.* 285 mμ and a lowering in the extinction coefficient to about half that shown by the free vitamin. In the extraction of the tocopherols from tissues considerable losses may occur.

The tocopherols have a maximum at *ca.* 294 mμ $E_{1\,\text{cm.}}^{1\%} = 100$ (Fig. 8). A wheat germ oil containing 0.5% tocopherol would show an $E_{1\,\text{cm.}}^{1\%}$

(294 mμ) = 0.5 due to the vitamin. A typical wheat germ oil might have $E^{1\%}_{1\,\text{cm.}}$ (294 mμ) = 2 which means that 75% of the absorption at 294 mμ is due to interfering absorbing substances. The direct spectroscopic determination is thus precluded in such cases.

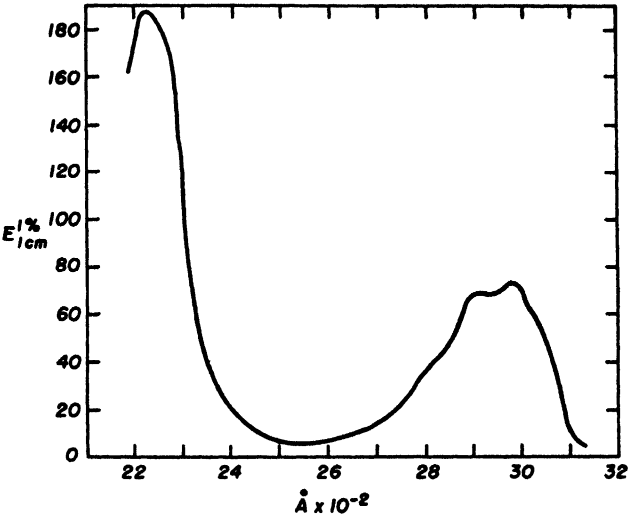

Fig. 8. Absorption curve of α-tocopherol in *iso*-octane (after Emerson *et al.* (202)).

In the estimation of tocopherols in livers, the vitamin A, which absorbs at 328 mμ and has $E^{1\%}_{1\,\text{cm.}} = 1725$, completely swamps the absorption due to the vitamin E. Carotenoids and ergosterol-like compounds also interfere.

If attempts are made to remove such interfering substances by saponification, losses are caused by the action of the alkali. Unless saponifications are carried out under very carefully controlled conditions, the losses of tocopherols can be very considerable.

Emmerie and Engel (203) have developed a method for the concentration of tocopherol from serum for use in assay procedures. Their method is as follows:

To 10 ml. of serum in a separatory funnel of 250 ml. capacity are added successively with gentle shaking after each addition:

1. 5 ml. of aqueous 0.2 *N* KOH.
2. 15 ml. of formaldehyde solution (37%), neutralized to phenolphthalein.
3. 15 ml. of ethanol.

After addition of 50 ml. of pure ether (peroxide-free), the contents of the separatory funnel are thoroughly shaken and the two layers are separated. After this first extraction with ether, the aqueous layer is extracted twice more with 50 ml. of ether, adding 10 ml. of ethanol to the aqueous layer each time before extraction with the ether. The combined ether extracts are washed successively with 25 ml. of 2% KOH and with 25 ml. of 1% H_2SO_4, after which the ether must be carefully washed acid-free with distilled water. These washings must be carried out by gently shaking the mixtures to prevent emulsification. The ethereal extract is dried over anhydrous Na_2SO_4 and evaporated *in vacuo* (after addition of 10 ml. of benzene to the ether extract) in an atmosphere of carbon dioxide. After evaporation, 10 ml. of benzene are added to the residue in the flask and the mixture again evaporated *in vacuo.* This addition of benzene is necessary to remove traces of water or ether which interfere with the adsorption, which must now take place, to remove vitamin A and carotenoids.

The residue in the flask (a very small drop) is taken up in 5 ml. of pure benzene and filtered through a Tswett column of purified Floridin XS earth (column of adsorbent 30 × 12 mm.). The earth is washed with 25 ml. of benzene and the filtrate is evaporated *in vacuo.* Acetyltocopherol behaves in the same way as tocopherol; it is not adsorbed from benzene solution by Floridin XS earth.

Emmerie (203A) has shown that stability of tocopherol may be influenced by the following factors during saponification of vitamin-containing oils, (1) the concentration of KOH, (2) an excess of KOH, (3) the saponification temperature, and (4) the duration of saponification.

The best procedure worked out by Emmerie for the saponification of tocopherol containing oils is as follows:

The saponifications were carried out in test-tubes (16 × 140 mm.) fitted with small reflux-condensers. After saponification, 8 ml. of absolute methanol and 10 ml. of water were added, and the mixture extracted three times with 50 ml. of peroxide-free ether. The combined ethereal extracts were washed with water, with dilute KOH solution (2%), and again with water until alkali-free. The extract was dried over anhydrous sodium sulphate, evaporated *in vacuo* in a carbon dioxide atmosphere and finally taken up in a suitable volume of ethanol or benzene (the latter is used when the absorption procedure with Floridin XS earth must be carried out). (See above.)

Moore and Rajagopal (204) have developed a method for the estimation of the tocopherols which has been applied to rat tissues. Their method is quoted below:

The rats were killed by coal gas. The tissues were dissected and digested by mincing and heating with 5% aqueous KOH. Digestion was usually complete in about 2 hr. Alcohol was then added to the digest and the fat extracted with ether. The extract was washed, evaporated, saponified for about 1 min. with hot alcoholic KOH and the unsaponifiable fraction separated in the usual way. Two precautions were necessary to prevent undue spectroscopic absorption in blank experiments. Ether of sp. gr. 0.730 (B.D.H.) which is satisfactory for use in vitamin A determinations by the $SbCl_3$ method (124) had to be redistilled. The use of anhydrous Na_2SO_4 in drying the ethereal extracts caused considerable absorption and was abandoned. In some experiments, the absorption was measured before saponification of the fat. In a few instances duplicate determinations were done on extracts prepared by grinding the tissues with anhydrous Na_2SO_4 and extracting by the Soxhlet method, the extracts being carefully washed to remove Na_2SO_4 before evaporation.

Calculation of Results. Absorption data have been calculated on the weight of fresh tissue, without reference to the fat content, and are expressed as $E^{100\%}_{1\text{ cm.}}$ instead of the usual $E^{1\%}_{1\text{ cm.}}$ in order to adjust the decimal point to a convenient position. Results are given to two figures, although the second is of doubtful significance if the first is high.

These authors conclude that the results obtained by this method are only qualitative and they suspect that their results are influenced by the saponification procedure used (*cf.* 203). Their results were in qualitative agreement with those obtained by the colorimetric method of Emmerie and Engel (205).

The direct spectroscopic method has been refined somewhat by making use of the fact that the oxidation products of the tocopherols (206), the so-called tocoquinones, absorb at 265 mμ and have $E^{1\%}_{1\text{ cm.}}$ about 4-5 times as great as that of the tocopherols. A method, making use of this fact, has been described by Cuthbertson, Ridgeway and Drummond (207). These authors are quoted below:

We have found it advisable, whenever relatively small amounts are likely to be present, to oxidize the unsaponifiable fraction with silver nitrate so that the tocopherols are quantitatively converted into the quinones, which show well-marked maxima in the vicinity of 265 mμ with an intensity about 4 times as great as that of the parent substances. This modification of the spectroscopic method enables one to detect as little as 0.01% of a tocopherol in an unsaponifiable fraction and to estimate with reasonable accuracy proportions above 0.03%. For oxidizing the unsaponifiable fractions, we employed a 5% solution of silver nitrate in 90% methyl alcohol acting at 65° C. for only 5 min. This method appears to have advantages over that employed by John (208) in giving a cleaner material and a better yield.

It appears that, unless pure or comparatively pure preparations of tocopherols are assayed by the spectroscopic method, the results may be very misleading.

6. VITAMIN K

The vitamins K_1 and K_2 and 2-methyl-1,4-naphthoquinone can be assayed by the spectroscopic method (209), provided they are very nearly pure, that absorbing impurities are not present, and that they are present in the oxidized quinone form and not as hydroquinones.

The absorption spectra of vitamin K_1 is shown in Fig. 9 and that of 2-methyl-1,4-naphthoquinone in Fig. 10.

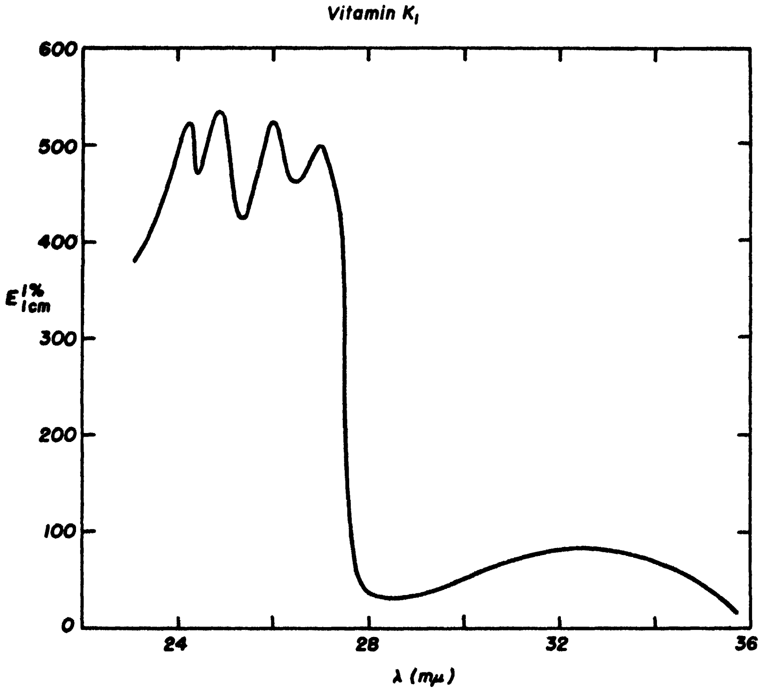

Fig. 9. Absorption curve of vitamin K_1 in hexane (after Morton (210)).

These compounds are determined by the comparison of the $E^{1\%}_{1\,cm}$ value at one of their maxima with that at this same wavelength for the pure vitamin.

7. VITAMIN C (ASCORBIC ACID)

In the determination of ascorbic acid by spectrophotometric methods, it is essential to use pure water free of copper ions (Cu^{++}). Mohler and

Lohr (211) have examined the effect of copper ions on the stability of the vitamin in aqueous solution and shown that, by adding small amounts of KCN, this effect can be eliminated.

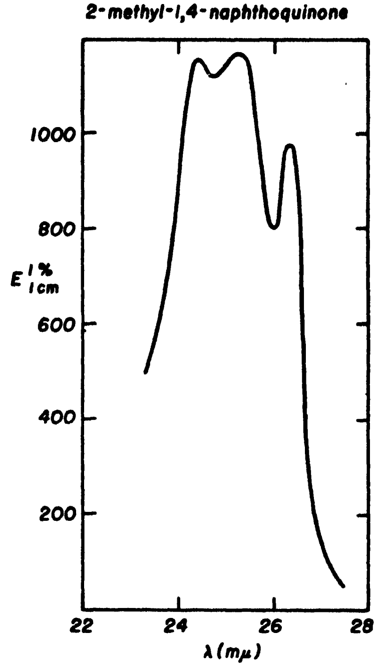

Fig. 10. Absorption curve for 2-methyl-1,4-naphthoquinone (after Morton (210)).

To obtain an accurate absorption spectrum of vitamin C, care must be taken not to expose the solutions to ultraviolet light for more than short periods, and to avoid oxidation. The absorption spectra of ascorbic acid (with an equimolecular amount of KCN) is shown in Fig. 11 (210). The following values (Table XVII) have been obtained for ascorbic acid in various solvents (80). It will be seen that the position of the absorption band is dependent on the solvent and is a function of the pH of the solution. Starzynski (212) has found that with decreasing pH the maximum of the absorption band of ascorbic acid shifts toward the shorter wavelengths.

Robertson (213) and Chevallier and Choron (214) have suggested methods based on the determination of the characteristic absorption spectra of ascorbic acid before and after destruction in a solution containing KCN. In the method of Chevallier and Choron (see also (215)),

TABLE XVII

Substance	Solvent	Conc. mg.-%	$\lambda_{max.}$	log E.
l-Ascorbic acid	H_2O	2.0	265	3.97
l-Ascorbic acid	H_2O and trace mineral acid	2.0	245	3.98
l-Ascorbic acid	EtOH	2.0	245	3.98
l-Ascorbic acid (Na Salt)	H_2O	2.0	265	4.04

1 g. of the tissue under examination is extracted, while grinding with 6 g. Na_2SO_4, with 20 ml. of alcohol which has been saturated with H_2S and containing 0.4 cc. of a solution of KCN (1/200) in water. After remaining for 30 minutes at 0° C. the mixture is filtered. An aliquot (2 ml.) is evaporated to dryness under N_2 below 60° C. The residue is

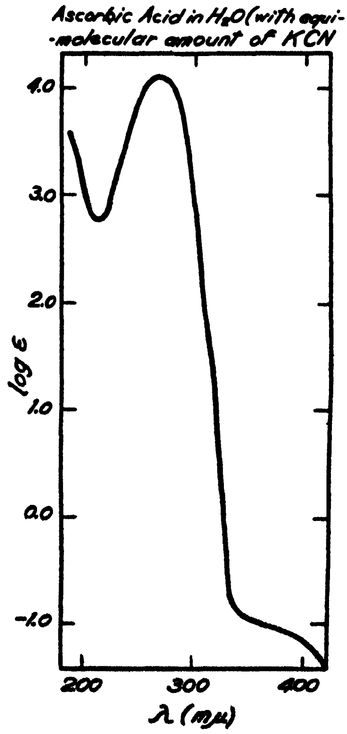

Fig. 11. Absorption curve for ascorbic acid in water (with equimolecular amount of KCN) (after Morton (210)).

taken up in 10 ml. of a 1/50,000 solution of KCN in water and the ultraviolet absorption curve determined between 240 and 300 mμ. Depending on the concentration of ascorbic acid present, this curve shows a maximum or an inflexion at 265 mμ. This solution is then irradiated until there is no longer any change in the intensity of the maximum. The difference between the intensities of the initial curve (A) and the irradiated curve (A‴) represents the absorption curve (B) for the ascorbic acid present. (See Fig. 12.) From the extinction coefficient of pure ascorbic acid measured under identical conditions the mg. of ascorbic acid/g. of tissue can be computed.

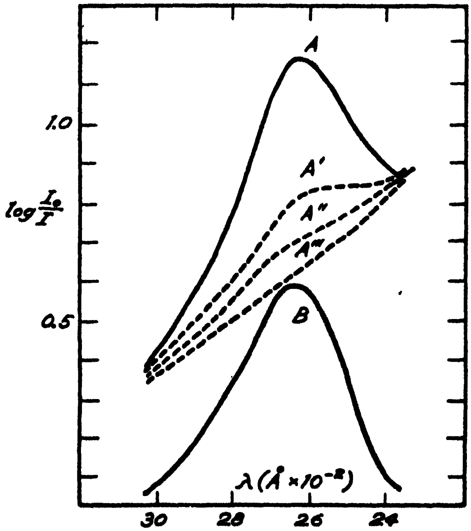

Fig. 12. Absorption curves of ascorbic acid extracted from tissue before (A) and after irradiation A‴. Curve B represents the absorption curve for the ascorbic acid present in the extract (after Chevallier and Choron (214)).

A somewhat different method has been suggested by Johnson (216). This method depends on the blank being obtained by the destruction of the ascorbic acid by oxidation in presence of Cu^{++} ions to dehydroascorbic acid, which has a negligible absorption.

The application of this method to the determination of ascorbic acid in aqueous humor is as follows (216):

In the spectrographic determination a dilution of [the aqueous humor] 1 in 10 generally sufficed to give the required concentration 0.5-2.5 mg. of ascorbic acid per 100 ml. Two equal volumes of humor, usually 1.0 ml., were necessary. One was immediately acidified with

0.2 N HCl and diluted with fresh glass-distilled water to give the desired concentration of humor in 0.02 N HCl. The ascorbic acid in the second sample was oxidized by diluting it with water to about 80% of its final volume and adding sufficient $CuSO_4$ solution to give ultimately a concentration of 2 mg. of Cu per litre of solution. At the reaction of these solutions, which was invariably neutral, the oxidation was complete in an hour. It was then acidified and the acid concentration adjusted as in the previous case. The absorption spectrum of each solution was measured by comparing the first with an equal depth of 0.02 N HCl and the second with a similar solution containing 2 mg. of Cu per litre.

The curve obtained with the oxidized solution represents the absorption due to non-vitamin C absorbing impurities. By subtracting the two curves, the absorption due to the ascorbic acid is found, and the concentration can be calculated from the curves obtained with solutions containing a known amount of the pure acid.

By this method, Johnson found good agreement with the results of the indophenol assay.

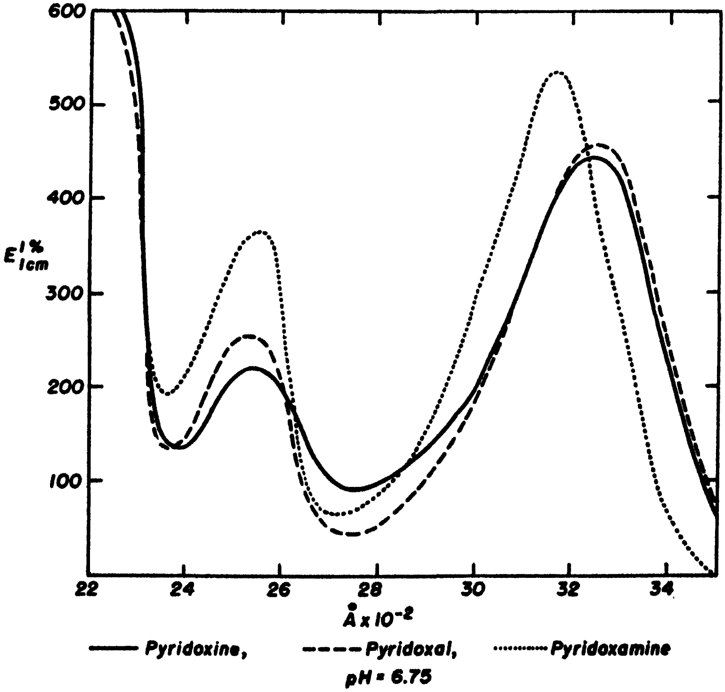

Fig. 13. Absorption spectra of pyridoxine, pyridoxal and pyridoxamine in water at pH 6.75 (after Melnick *et al.* (219)).

8. VITAMIN B_6 (PYRIDOXINE, PYRIDOXAL, AND PYRIDOXAMINE)

Pyridoxine shows selective absorption in the ultraviolet region of the spectrum. This absorption spectrum shows marked and characteristic changes with change of hydrogen ion concentration (217, 218).

Melnick *et al.* (219) have recently shown that pyridoxamine and pyridoxal have very similar absorption spectra and show the same changes with change of hydrogen ion concentration.

These authors summarize their findings as follows:

The ultraviolet absorption curves show no maxima common to all three compounds (Fig. 13). However, if readings are taken at 325 mμ of solutions at pH 6.75, close estimates of the total amounts of the compounds present may be obtained, despite variations in their relative concentration (see Fig. 13). At that wavelength, there is an absorption maximum common to pyridoxine and pyridoxamine, and while pyridoxal absorbs approximately 20% more light at its 315 mμ maximum, at 325 mμ all three compounds absorb to the same extent. The $E_{1\,\mathrm{cm.}}^{1\%}$ values at 325 mμ in pure solution at pH 6.75 all approximate 440 ± 1.5%, expressed in terms of the free bases. Spectrophotometric analyses, of course, cannot be applied, unless the test solutions are free from irrelevant light-absorbing materials, or unless blank solutions containing none of these vitamin B_6 factors are available for evaluating the interference.

III. Methods Involving Fluorimetric Determinations

1. GENERAL

The phenomenon of fluorescence occurs when a substance absorbs radiant energy and then re-emits it as radiant energy of a different wavelength. In most cases, the emitted radiation is of a longer wavelength than the exciting radiation, but this is not universally true (*e.g.,* the resonance fluorescence of certain dyes in the mesomorphic state). The fluorescent absorption bands given by such excited substances are characteristic for them and do not depend on the wavelength of the exciting radiation.

The quantitative measurement of fluorescence is known as fluorimetry, and is concerned with the determination of the concentration of fluorescent substances in solution.

The intensity of light emitted by a fluorescent substance is a simple function of the concentration of the substance, under certain circumstances, and thus can be used for quantitative estimation.

For the analytical measurement of fluorescence it is the object to excite the molecule with radiation at the wavelength of maximum absorption. Such light is isolated by the requisite filters from other wavelengths. The emitted radiation is thus measured, or compared, with that of some standard light, freed from reflected incident light, by means of filters or a monochromator.

At low concentration the ratio of the intensity of the emitted fluorescent radiation is proportional to the incident light absorbed by the molecule.

When the concentration of the absorbing molecule increases, the intensity of the emitted radiation is no longer proportional to the absorbed light. This effect is known as "concentration quenching." Other factors may cause such "quenching" of fluorescence.

The fluorescence of a substance in solution has been shown by Vavilov (220) to be a function of the absolute temperature, other variables remaining constant. Vavilov has also demonstrated that fluorescence can be "quenched" by foreign molecules in the fluorescing solution (221). Weil-Malherbe and Weiss (222) have pointed out that oxygen at 1 atmosphere pressure reduced the fluorescence of thiochrome in isobutanol solution by 27.5%, and that of quinine sulfate, 17.5%. The effect of electrolytes on the fluorescence has been examined by Stoughton and Rollefson (223, 224, 225) and by Ellinger and Holden (226). The latter authors have also examined the effect of pH on the fluorescence of thiochrome. Some suggestions have been put forward for overcoming the quenching effect of oxygen and the effects of temperature in the thiochrome assay (227). For a more thorough discussion of the phenomena of quenching the reader is referred to (228).

Since the discovery of numerous fluorescent pigments of biological importance, methods of fluorimetry have been developed for the estimation of such compounds.

Desha (229) was the first to make quantitative measurements for the estimation of the amount of fluorescent pigments in solution. Since then, numerous methods and instruments have been developed for use in routine fluorimetry in biochemical work (228).

2. THE FLUORIMETRIC ASSAY OF VITAMIN A ESTERS

Vitamin A esters in alcoholic solution, upon irradiation with ultraviolet light, show an initial steep increase in fluorescence followed by the complete destruction of the vitamin over a prolonged period of time (230). In nonpolar solvents, the initial increase in fluorescence is not found, but a small decrease is sometimes found. The destruction of the vitamin is very much slower in these solvents on prolonged irradiation.

The fluorescence of vitamin A esters in both types of solvents is proportional to its concentration. Free vitamin A alcohol fluoresces only weakly and does not show the initial increase in fluorescence in alcohol. Vitamin A_2 concentrates show similar fluorescent characteristics as those of vitamin A.

This difference between the vitamin alcohol and its esters was made the basis for the assay of vitamin A esters in fish liver oils by Sobotka, Kann and Winternitz (231). The results obtained by this method were not, however, in agreement with the results obtained in analytical molecular distillation experiments (109), or by distribution between 83% aqueous alcohol and petroleum ether. Both these methods showed that, in fish liver oils, less than 5% of the vitamin A was present as the free alcohol, whereas the fluorimetric method had shown considerably less esters present.

Kascher and Baxter (109), in discussing these results, pointed out that the fluorimetric standard used by Sobotka *et al.* (vitamin A acetate) is not equivalent, fluorimetrically, to the naturally occurring vitamin esters.

3. THE FLUORESCENT MICROSCOPIC DEMONSTRATION OF VITAMIN A IN TISSUE

BY HANS POPPER *

The green, markedly ultraviolet-sensitive, fluorescence of vitamin A can be utilized for its histologic demonstration (85, 86, 87). In frozen sections of organs in which vitamin A can be demonstrated by biologic assay or chemical methods, a green, quickly fading fluorescence is noted under the fluorescence microscope, which is localized chiefly in intracellular lipide structures. The degree of fluorescence depends roughly on the amount of vitamin A present. The greater the presence of the vitamin A at one site, the slower the fading. The fluorescent microscopic procedure is, therefore, primarily used for localization of vitamin A in the tissue. It permits a rough estimation of the amount present, but not a quantitative assay.

A. TECHNIQUE

a. Microscope. In the fluorescent microscopic demonstration of vitamin A, a light source rich in ultraviolet light is used. This is best achieved with a mercury vapor bulb. The greater part of the visible

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*** The author wishes to acknowledge his indebtedness to Dr. Hans Popper, Director of the Hektoen Institute for Medical Research, Chicago, Ill., for contributing the section on "The Fluorescent Microscopic Demonstration of Vitamin A in Tissue" for this chapter.**

light is removed by an ultraviolet filter such as the Corning glass color filter no. 584. The residue of the visible light, chiefly of red character, is absorbed either by a glass cell containing a 5% $CuSo_4$ solution, or by a second Corning glass color filter No. 428. The ultraviolet rays eliciting the vitamin A fluorescence are in the longer range of the ultraviolet and are thus not greatly absorbed by glass. Consequently, a standard microscope with the usual Abbe condenser is employed. The mirror has preferably an aluminum coating. The ultraviolet rays which have not been transformed into visible light by the fluorescent material are absorbed by a filter inserted into the eye piece, such as a Leitz ultraviolet protecting filter No. 8574-A. With the described filter system, a dark field is seen in which the tissue constituents are recognized by their fluorescence. This permits an easy orientation in an unstained section.

b. Preparation of Material. The vitamin A fluorescence in tissues is unstable in intact organs as well as in fixatives of a 5% formalin. The material is examined as soon as possible after it becomes available. Human organs are kept in the fixative not more than 18 hr., animal organs not more than 8 hr. Since vitamin A is fat-soluble, frozen sections are used. Most of the embedding methods, like the paraffin or celloidin techniques, require too much time and the use of fat solvents. The sections are mounted in water and should be examined immediately. Since other mounting media are not suitable, no permanent sections can be made. For permanent records, fluorescence photomicrographs are used, which should be taken by means of a camera with periscope view finder attachment.

c. Observation. Any green fading fluorescence is considered vitamin A fluorescence. It is usually, but not always, located in fatty material. However, not all fatty material in tissues reveals vitamin A fluorescence. For exact localization, it may be helpful to stain the sections with 1% aqueous methylene blue for 30 sec. This stain interferes only slightly with the vitamin A fluorescence. If the ultraviolet filter (Corning No. 584) is changed to a ground glass filter, the specimen can be observed in visible light.

B. SPECIFICITY OF THE METHOD

The following, partly suggestive and partly conclusive evidence, has been presented for the assumption that the green quickly-fading fluorescence in tissue sections is due to the presence of vitamin A.

a) The visual appearance of the fluorescence under the microscope is identical with that of droplets of an oil-in-water emulsion containing pure vitamin A. Spectroscopic analysis of the fluorescent microscopic picture thus far has not succeeded because of the quick fading of the vitamin A fluorescence.

b) The fluorescence in tissue section is abolished by reagents which remove or destroy vitamin A and is left intact by reagents which have no influence on vitamin A.

c) The distribution of the vitamin A fluorescence in animal or human organs agrees with that found by chemical, spectroscopic, or biologic assays.

d) The most conclusive evidence is the fact that in rats kept on vitamin A-deficient diets, the characteristic green fading fluorescence disappears parallel with the decrease of the vitamin A storage demonstrated by chemical method. Deficiencies in other vitamins fail to produce this effect. Upon repletion of those rats with vitamin A, the fluorescence reappears in typical distribution. The amount which then appears agrees with the dose of vitamin A fed and the concentration chemically

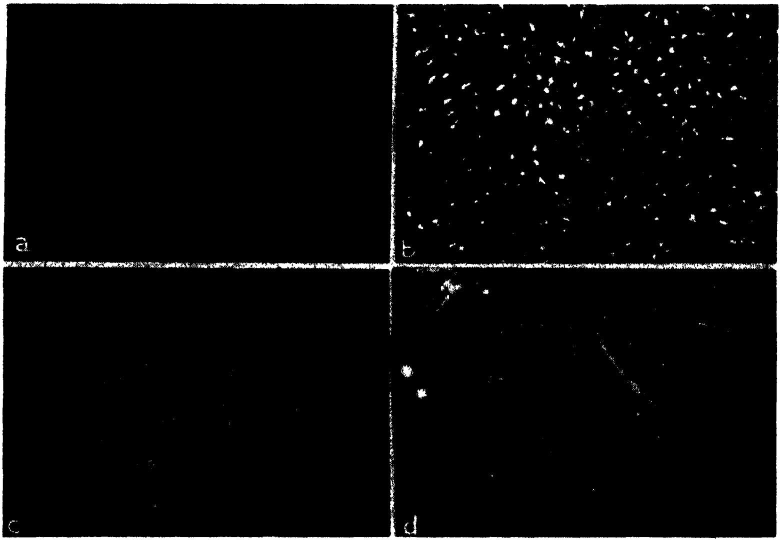


Fig. 14. Fluorescence Photomicrographs. A. Liver of vitamin A-deficient rat without any vitamin A fluorescence. Shadows of a vessel seen. B. Liver of a vitamin A deficient rat after feeding of large dose of vitamin A. Vitamin A fluorescence is revealed by Kupffer cells and small fat droplets on the edge of the liver cells. C. Normal human liver. Moderate amounts of vitamin A fluorescence imparted by Kupffer cells and fat droplets on the edge of the epithelial liver cells, especially marked in the center of the lobule around central vein. D. Jejunum of a rat after feeding of a large dose of vitamin A. Vitamin A fluorescence is seen in epithelial cells of the mucosa and in the cells and lymphatics within the villi.

demonstrable in the liver. No other substance fed to vitamin A-deficient rats, with the exception of carotene, reproduces the fluorescence.

Therefore, the evidence that the green fading fluorescence in sections is due to the vitamin A appearing conclusive for rats, seems strongly suggestive for other animals and humans. In the latter group, reference to vitamin A fluorescence implies that it can be caused by vitamin A, but does not exclude the possibility that other substances, thus far unknown, may be responsible for it.

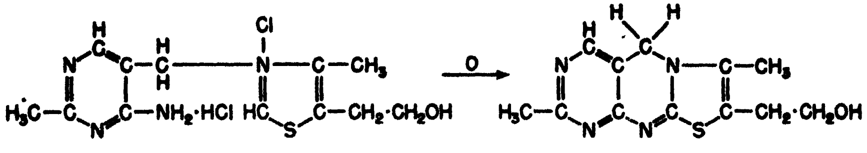
C. DISTRIBUTION OF VITAMIN A FLUORESCENCE

Vitamin A fluorescence has been demonstrated in various human and animal organs. It was especially studied in the liver, where characteristic changes appear in pathologic conditions. It is seen in the intestine during absorption of vitamin A. It is visible in the adrenal cortex, especially in the layer which apparently produces corticosterone, in the testicle, and in the ovary where it undergoes characteristic changes during the menstrual cycle and during pregnancy. It is noted in lactating breasts, in fat tissue, in the retina, depending upon the stage of visual adaptation, and in certain pathologic conditions of the kidneys. It may be found in tumors deriving from a mother tissue which reveals vitamin A fluorescence. Extremely high amounts are seen in the liver of salt-water fish. In the livers of fresh-water fish, a yellow-brown, slowly fading fluorescence may be noted which is due to vitamin A_2.

4. THE FLUORIMETRIC ASSAY OF THIAMINE

A. INTRODUCTION

Thiamine (I), on oxidation, is converted into the yellow pigment thiochrome (II), which shows an intense blue fluorescence when excited by ultraviolet light. (*e.g.*, the 365 mμ mercury arc line) (232).



I

II (Thiochrome)

Many oxidizing agents can be used for this conversion, but potassium ferricyanide (234) in alkaline solution (pH 10.0) is the oxiziding agent of choice in fluorimetric assay methods based on the production of thiochrome.

The assay of thiamine by this method was first proposed by Jansen

(235), who extracted the thiochrome from the oxidized extracts by means of isobutanol and determined the concentration of the thiochrome fluorimetrically. Many investigators (236, 237, 238, 239, 240, 241, 242, 243) have used this method with little modification.

In many cases, interfering fluorescent substances are present in the isobutanol extracts and attempts have been made to rid extracts of these by absorption on a variety of absorbents such as Frankonite. These methods were not too successful, and it was not until Hennessy and Cerecedo (244) examined this problem, that a satisfactory method was worked out. These authors (245, 246, 247) had previously shown that thiamine could be isolated very readily from a variety of natural sources by a base-exchange technique using the synthetic zeolite Decalso at an acid pH. The vitamin could be readily eluted by means of a solution of NH_4NO_3.

In 1939, these authors showed that this method could be applied very successfully to the fluorimetric assay of thiamine in order to eliminate the interfering substances.

The assay of Jansen (235) determined only the free thiamine in samples and did not take into account the thiamine present in the form of its pyrophosphoric ester (cocarboxylase), which has an equivalent biological activity to thiamine. In many animal tissues and yeasts the thiamine is present almost entirely in the form of its ester. Cocarboxylase can be oxidized to a phosphoric ester of thiochrome which cannot be extracted by means of isobutanol (248). The fluorimetric assay of such combined thiochrome is very unreliable because of interfering substances in the aqueous layer. Attempts have been made to get around this difficulty by enzymatic fission of the cocarboxylase before oxidation. Tauber (249, 250, 251), and Hennessy and Cerecedo (244), have used an enzyme preparation from kidney tissue, whereas Lohmann and Schuster (252) have used, beside the kidney phosphatase, an enzyme from prostatic tissue. Yeast powder (253) and a yeast glycerol extract (254) have also been used.

Takadiastase (255, 256, 257), mylase (255, 256), clarase (255, 256), and polidase (255), have been used for the liberation of thiamine, particularly in extracts of starchy materials (for a comparison of several of these enzyme the reader is referred to (255)).

Hennessy *et al.* (258) have compared several commercial enzyme preparations and have pointed out that aluminum, mercuric and ferric ions inhibit the enzymatic hydrolysis of the cocarboxylase, whereas ferrous ions have the opposite effect.

Andrews and Nordgren (259) have suggested that some of the difficulties in determining thiamine in bread may be due to the synthesis of cocarboxylase from the thiamine by the action of yeast. Lipton *et al.* (260) have shown that yeast can couple thiamine and phosphoric acid to give cocarboxylase.

In cow milk it has been proposed (261) to use a mixture of takadiastase and a proteolytic enzyme, *e.g.*, papain, to free the thiamine from a protein complex.

B. THE METHOD OF HENNESSY AND CERECEDO

Hennessy and Cerecedo (244) have developed the method which is in almost universal use for the assay of thiamine and cocarboxylase. Their method is as follows:

Preparation of the Extracts. The finely divided sample is refluxed for 3 min. with 5-20 parts of 2% acetic acid solution. After cooling and centrifuging, the residue is reëxtracted and the filtrates combined. In case of gelation, where good separation is not accomplished by centrifuging, the solution is made 30% with alcohol. If the extract is to be used in Method II or Method III (*cf.* below), the alcohol is later removed by vacuum distillation and the volume made up with water. In most cases the extract does not represent the total volume of acetic acid solution added. Correction is made for this in the calculation. Liquids such as milk, blood, and animal tissue extracts are prepared for analysis by precipitation of the proteins with just enough 2% trichloroacetic acid solution, refluxing of the resulting suspension, and centrifuging after cooling.

In the case of adsorbates such as the International Standard or the U. S. P. Reference Standard, approximately 100 mg. is extracted with four 5 cc. portions of boiling 2 *N* HCl. The residue is separated each time by centrifuging. Finally, the residue is extracted twice by mixing with 10 cc. of cold 2 *N* NaOH. The acid and alkaline eluates are combined and, after adjustment to the required pH, the volume is made up to 50 cc. with water.

Concentrates which are water soluble are merely diluted to a convenient volume.

In the case of urine, the sample is adjusted to the proper acidity and assayed by Method II.

The Assay. As we perform the thiochrome assay, there are three alternate procedures: direct assay (Method I), assay following base-exchange (Method II), and assay following enzymatic hydrolysis with or without base-exchange (Method III). Method I is used whenever the blank is comparatively small and the recovery of vitamin, added in an equal quantity to that present, is practically quantitative. Further, there should be left in the aqueous layer following the isobutanol extraction not more fluorescence, as observed visually, than is in the same layer of the blank or of a sample containing an equal quantity of pure thiamine. If direct assay is not practical, though no unusual unextractable fluorescence is noted, Method II is used. When obvious residual fluorescence is noticed in the alkaline aqueous layer, then Method III is employed.

Method I. Such a volume of solution as will contain 0.2-20.0 γ of the vitamin is made up to 5 cc. in a 25 cc. glass-stoppered graduated cylinder. To the sample 0.05-0.20 cc. of 1% potassium ferricyanide solution is added. Three cc. of 15% NaOH solution is added with mixing and immediately followed by 13 cc. of isobutanol. The mixture is shaken vigorously for 1 min. and centrifuged for 0.5 min. to separate the two layers. It is then carefully poured into a separatory funnel, and the lower layer removed and set aside for comparison with the same solution in the blank determination to ascertain whether Method III be necessary. The isobutanol solution is transferred to a test tube and 2-4 g. of anhydrous sodium sulfate is allowed to settle and 10 cc. of the perfectly clear isobutanol solution is decanted into the cuvette of the fluorimeter. The same procedure is repeated for the blank, except that potassium ferricyanide is omitted, and the order of addition of the NaOH and the isobutanol is reversed.

When thiamine chloride is added to a sample in an amount equal to that already present, 93-102% is recovered in this method.

Method II. *The Exchange Tube.* (Obtained from E. Machlett and Son, N.Y.C.). A tube of 7 mm. inside diameter surrounded by an outer jacket over 40 cm. of its length is used. A constriction at the lower end of the jacketed portion holds a plug of glass wool. On this is filled a 20 cm. column of 30-mesh Decalso (obtained from the Permutit Co., N.Y.C.). At the upper end of the tube is a funnel for introduction of liquids, at the lower end a two-way stopcock for gravity flow and suction.

When newly introduced, the Decalso is freed of excess alkali and converted to the potassium form by alternately passing through four 15 cc. portions each of boiling 2% acetic acid and 25% potassium chloride solution. During this treatment steam is passed through the outer jacket to keep the temperature near 100° C. The column is then washed by passage of three 15 cc. portions of boiling water, which leaves the tube ready for use.

The Exchange. From 5 to 20 cc. of the solution containing up to 10.0 γ of thiamine as indicated in a rough direct assay is adjusted to pH 4.0-4.5 with acetic acid. It is brought to a boil and introduced into the exchange tube which is kept near 100° C. The solution, which is allowed to pass through the Decalso bed in 3-5 min., is followed by three 5 cc.

portions of boiling water. The stopcock is shifted to suction and the excess water drawn from the tube.

For the removal of the vitamin from the Decalso bed, boiling 25% KCl solution is introduced into the funnel at the top of the exchange tube. The flow of the solution through the Decalso bed should be near 1 cc./min., 10-25 cc. being collected. The larger volume is used when the sample contains the larger quantities of thiamine. For 0.1 γ, 20 cc., and for 7-10 γ, 25 cc. The approximate content of the sample can be judged, after some experience, by visual observation of the oxidized sample in ultraviolet light. Aliquots of the KCl effluent (usually 5 cc.) are assayed by Method I. The amount of the 1% $K_3Fe(CN)_6$ solution required is 0.05 cc.

Since the Decalso tube may be used repeatedly by merely washing with hot water for removal of the KCl left in the tube, it is essential, when a sample of low thiamine content follows a sample of high content, that all the thiamine of the former sample be removed. Refilling the exchange tube is resorted to when a determination on a pure sample of thiamine shows either a large blank or a low recovery. Recovery of thiamine chloride added to samples is 91-97% when Method II is used.

Method III. *Preparation of the Enzyme.* Ground defatted beef kidney is extracted by shaking or stirring with an equal weight of 1% NaCl solution for 2 hr. The mixture is centrifuged and the centrifugate put aside in the refrigerator. The residue is re-extracted in the same way. The combined extracts are stirred with sufficient 300-mesh Volclay (obtained from the American Colloid Co., Chicago, Ill.) to give a thin paste, which is centrifuged. On filtration of the centrifugate, a clear liquid is obtained, the volume of which is about 60% of that of the original extract. One-half volume of acetone is stirred into the extract and the precipitate removed by centrifuging. A second portion of acetone, equal in volume to that already added, is stirred into the centrifugate. The precipitate which forms is collected by centrifuging and is dried in a vacuum desiccator over sulfuric acid. The dry residue is powdered and preserved in a brown stoppered bottle.

Enzymatic Hydrolysis. The solution to be tested is adjusted to pH 6.5-7.0, 5-20 cc. mixed with 20-80 mg. of the powdered enzyme and kept at 37° C. for 3 hr. The hydrolyzate is acidified by the addition of one drop of acetic acid, boiled, cooled, and centrifuged. An aliquot of the centrifugate is assayed by Method I or, if necessary, Method II.

The recovery of thiamine chloride and of cocarboxylase is 90-97% when added to samples assayed by Method III.

Fluorimetry. In operating the fluorimeter,* the incident beam passes through the thiochrome solution, causing it to fluoresce. The fluorescent light strikes a photocell at the side of the cuvette and is transformed into electrical energy, the current of which is measured by a galvanometer. With a constant incident intensity, there is a linear relationship between the deflection of the galvanometer and the thiamine chloride up to 20 γ.

The instrument is calibrated by the following procedure. With a fully opened iris diaphragm in the path of the incident ultraviolet light, Q, T, and B, respectively, are the galvanometer deflections measuring the fluorescence of a standard quinine sulfate solution in 0.1 N H_2SO_4, of a thiochrome solution prepared according to Method I from N γ of thiamine chloride and of isobutanol obtained in the same way without any thiamine chloride being used. With Q_o as the quinine deflection for a lower incident intensity, the deflection/γ of thiamine chloride is

$$Q_o = \frac{T - B}{NQ}$$

A photocell at the rear of the empty cuvette allows measurement of the incident intensity. These deflections plotted against the various readings for Q_o may be used conveniently to set the deflection/γ of thiamine chloride. In the measurement of the unknown, the difference between the deflections induced by the oxidized and unoxidized aliquots is divided by the deflection/γ of thiamine chloride as set directly by reference to Q_o, or indirectly to the rear photocell deflection just before pouring the thiochrome solution into the cuvette.

The results obtained by this method, when applied to a large variety of thiamine sources, gave results in good agreement with biological results.

The method has been shortened by suggestions made by Hennessy (262). These modifications are quoted below:

Preparation of Extracts. The extraction is now accomplished in a single treatment of the sample with 0.1 N H_2SO_4. The pH of 4.0-4.5, required for the enzymatic hydrolysis and for the base exchange, is attained by the addition of 0.1 volume of 1.2 M sodium acetate solution to the extraction mixture.

Method I. All preparations, including the standard thiamine chloride, should be in 20-25% KCl solution at the oxidation step, to keep the volume of isobutanol constant in the extraction. It has been found advantageous to mix the ferricyanide and the alkali before adding them to the sample. A separatory-centrifuge tube (obtainable from E. Machlett and Son, N. Y.) has been devised so that oxidation, extraction, separation, and clarification may be carried out in this single vessel with uniform timing throughout. The shaking time for the isobutanol extraction seems best at 1.5 min., followed by centrifuging at 500 r.p.m. for 0.75 min.

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* A Pfaltz and Bauer fluorimeter was used.

Method II. *Exchange Tube.* The tubes for the exchange are 15 × 0.7 cm. with a 25 cc. reservoir at the upper and a 3.0 × 0.03 cm. capillary at the lower end. A 6-7 cm. column of Decalso is held in the tube by a small plug of glass wool at the lower end.

The Exchange. The base exchange is conducted at room temperature, using 60- to 80-mesh Decalso prepared in bulk by stirring or shaking with four 10-volume portions of 3% acetic acid for 10 min. each. Between the second and third acid washing a 15 min. treatment with 5 volumes of 25% KCl solution is introduced. Thorough washing with water finishes the preparation of the Decalso. The eluting mixture is 25% KCl in 0.1 *N* HCl. Complete removal of thiamine from the zeolite is accomplished by passing 2 cc. of this mixture at room temperature at a rate not exceeding 0.9 cc./min. This allows for four 5 cc. aliquots. As many as 16 of these tubes may be run in parallel and 8 samples carried through this step in duplicate in 70 min. Although the Decalso may be used repeatedly, it is safer practice to use a fresh charge for each sample.

Method III. *Enzymatic Hydrolysis.* The enzymatic hydrolysis is brought about in 2 hr. at 50° C. using 0.5% takadiastase at pH 4.0 to 4.5. This hydrolysis, if carried out on extraction mixtures, is done before any separation of the still insoluble portions is attempted.

Fluorimetry. The use of quinine sulfate is to be preferred to the use of the rear photocell of the fluorophotometer for setting the galvanometer deflection/γ of thiamine chloride. The quinine sulfate fluorescence should be determined immediately before that of the unknown.

The method of Hennessy and Cerecedo for the estimation of thiamine has been used extensively with only minor modifications to suit specific uses (263, 264, 265, 266). A micro-method for use in assaying very low potency samples has been worked out (267) but the accuracy is only about ± 20% at levels of 0.001 mg., the accuracy increases to ± 3% at 0.05 mg.

C. THE METHOD OF THE U. S. PHARMACOPOEIA XIII (268)

The method of the U. S. Pharmacopoeia is quoted below:

Reagents. *Double-Normal Sodium Acetate.* Dissolve 275 g. of reagent sodium acetate in sufficient distilled water to make 1000 cc.

Bromocresol Green and Thymol Blue pH Indicators. Triturate 100 mg. of the indicator in an agate mortar with the volume of *N*/20 NaOH specified in the following table, or, with its equivalent of *N*/50 NaOH.

When the indicator has dissolved, the solution is diluted with carbon dioxide-free water to make 200 cc. (0.05%).

The solutions should be kept in stoppered bottles and protected from light.

	pH range	Molecular Weight	Color change	Solubilizer
Bromocresol green	3.8-5.4	699	yellow-blue	7.2 cc. $N/20$ NaOH
Thymol blue	8.0-9.6	466	yellow-blue	4.3 cc. $N/20$ NaOH

Enzyme Solution. Prepare, on the day on which it is to be used, a 10% solution in distilled water of an enzyme preparation potent in diastatic and phosphorolytic activity.

Base Exchange Silicate. Purify an artificially prepared silicate of the base-exchange type, in the form of a granular powder of 50- to 80-mesh size, in the following manner: Place a convenient quantity (100-500 g.) of the base exchange silicate in a suitable beaker, add sufficient hot 3% acetic acid ($HC_2H_3O_2$) to cover the material, and boil for 10-15 min., stirring frequently. Allow to settle, and decant the supernatant liquid. Repeat this washing three times. Then wash in a similar manner three times with a hot 25% aqueous solution of KCl (1 part by weight of KCl in 4 volumes of solution), and finally wash with boiling distilled water until the last washing gives no reaction for chloride. Dry the material at approximately 100° C. and store in a well-closed container.

Base Exchange Tube. The base exchange glass tube has an overall length of 200 mm. A reservoir at the upper end is 50 mm. in length and 25 mm. in diameter. This converges into the adsorption tube which is 5-6 mm. in internal diameter and approximately 140 mm. long. At the lower end, the tube is drawn into a capillary approximately 10 mm. long and of such diameter that, when the tube is charged, the rate of flow will not be more than 1 cc./min.

Prepare the tube for use by placing over the upper end of the capillary, with the aid of a glass rod, a pledget of fine glass wool. Add a distilled water suspension of 1.0-2.0 g. of the purified base exchange silicate to the adsorption tube, taking care to wash down all of the silicate from the walls of the reservoir. To keep air out of the adsorption column, a layer of liquid must be kept above the surface of the silicate throughout the adsorption process and the tube may be prevented from draining by placing a rubber cap (filled with water to avoid inclusion of air) over the lower end of the capillary.

Neutral Potassium Chloride Solution. Dissolve 250 g. of reagent KCl in sufficient distilled water to make 1000 cc.

Acid Potassium Chloride Solution. Add 8.5 cc. of reagent HCl to 1000 cc. of the neutral KCl solution.

Sodium Hydroxide Solution, 15%. Dissolve 15 g. of NaOH in sufficient distilled water to make 100 cc.

Potassium Ferricyanide Solution, 1%. Dissolve 1 g. of reagent $K_3Fe(CN)_6$ in sufficient distilled water to make 100 cc. This solution must be freshly prepared on the day it is used.

Oxidizing Reagent. Prepare the solution by mixing 4.0 cc. of the 1% $K_3Fe(CN)_6$ solution with sufficient of the 15% NaOH solution to make 100 cc. This solution should be used within 4 hr.

Quinine Sulfate Stock Solution. Quinine sulfate solution is used to govern the reproducibility of the fluorophotometer. A stock solution of quinine sulfate is prepared by dissolving 10 mg. of quinine sulfate in sufficient 0.1 *N* H_2SO_4 to make 1000 cc. Preserve this solution in light-resistant containers.

Quinine Sulfate Standard Solution. Dilute 1 volume of the quinine sulfate stock solution with 39 volumes of 0.1 *N* H_2SO_4. The solution fluoresces to approximately the same degree as the thiochrome obtained from 1 γ of thiamine hydrochloride. Preserve this solution in light-resistant containers.

Thiamine Hydrochloride Stock Solution. Weigh accurately 20-25 mg. of U.S.P. Thiamine Hydrochloride Reference Standard which has been kept in a desiccator over phosphorus pentoxide for at least 16 hr. Since the Reference Standard is hygroscopic, precautions must be taken to avoid absorption of moisture. Dissolve in 20% alcohol adjusted to a pH of 3.5-4.3 with HCl and make up to a volume of 1000 cc. Store in a cool place in a well-closed, light-resistant container.

Thiamine Hydrochloride Standard Solution. From a portion of the stock solution that has been warmed to room temperature, transfer to a 100 cc. volumetric flask an aliquot containing exactly 100 γ of thiamine hydrochloride, and dilute to 100 cc. with distilled water adjusted to a pH of 3.5-4.3 with HCl. Each cc. of this solution contains 1 γ of thiamine hydrochloride.

Dilutions of this solution are treated in the same manner as that used in the *Preparation of Assay Solution* with respect to acid digestion, enzyme treatment, adsorption, and elution from the base exchange silicate.

Preparation of Assay Solution. The amount of material taken for the assay should be such that the ratio of the volume of 0.1 *N* H_2SO_4 used for the extraction to the quantity of sample is at least 15 to 1, and the content of thiamine equivalent to 30-100 γ of thiamine hydrochloride. Place the accurately weighed quantity of the material to be assayed in 65 cc. of 0.1 *N* H_2SO_4 contained in a 100 cc. centrifuge tube and digest it on a steam bath with frequent mixing for 30 min. The liquid must remain acid during the digestion. If not distinctly acid to the thymol blue pH indicator, add sufficient diluted H_2SO_4 to make it acid. Cool, and adjust the pH to between 4 and 4.5 by the addition of double-normal sodium acetate solution, using bromocresol green pH indicator as an external indicator in conjunction with a spot plate. Add 5 cc. of the enzyme solution, mix, and incubate at 45-50° C. for 3 hr. Cool, centrifuge the mixture until the supernatant liquid is clear or practically so, and quantitatively transfer the supernatent liquid to a 100° cc. volumetric flask. Wash the residue by centrifuging with 10 cc., then with 5 cc. of 0.1 N H_2SO_4. Add the washings to the supernatant liquid and dilute to 100 cc. with distilled water.

Pass through the prepared base exchange tube an aliquot of the solution estimated to contain 5-10 γ of thiamine, wash the tube with three 5 cc. portions of boiling, distilled water, taking care to prevent the surface of the liquid from falling below the surface of the silicate.

Elute the thiamine from the *base exchange silicate* by passing successively through the tube small portions of hot *acid potassium chloride solution*. Collect the first 15 cc. of the liquid (*eluate*) in a glass-stoppered, 25 cc. volumetric flask, cool, and dilute to a volume of 25 cc. with acid potassium chloride solution. *This constitutes the assay solution.*

Oxidation of Thiamine to Thiochrome and Measurement of Fluorescence. Determine the thiamine content of the oxidized assay solution by comparing the intensity of fluorescence of an extract of this solution exposed to ultraviolet rays ranging from 350 mμ to 400 mμ with that from oxidized Thiamine Hydrochloride Standard Solution. The intensity of the fluorescence is proportional to the quantity of thiamine present and may be measured with the aid of various instruments.

To oxidize thiamine to thiochrome, add to quantities of the assay solution and of the similarly treated Thiamine Hydrochloride Standard Solution containing 0.10-2 γ of thiamine, sufficient acid potassium chloride solution to produce a volume of 5 cc. Then add, with mixing, 3 cc. of oxidizing solution. Within 2 min. add 13 cc. of isobutyl alcohol and shake vigorously for at least 1.5 min. Centrifuge the mixture at a low speed until a clear supernatant solution is obtained. Measure in a fluorimeter the intensity of fluorescence of the isobutyl alcohol solution directly if clear, or, if cloudy, after shaking with 2 g. of anhydrous sodium sulfate. Compare with this the intensity of fluorescence produced after oxidation of the properly prepared Thiamine Hydrochloride Standard Solution. Quinine Sulfate Standard Solution is used to govern the reproducibility of the instrument. Correction must be made for fluorescence produced by substances other than thiamine by determining the intensity of fluorescence of Thiamine Hydrochloride Standard Solution, and assay solutions treated as described above, but with 15% NaOH solution replacing the oxidizing reagent.

The Thiochrome Assay for Thiamine Hydrochloride is applicable to substances such as Thiamine Hydrochloride, and a number of other products, but cannot be relied upon when certain interfering substances are present; in the latter case, use the *Biological Assay for Thiamine Hydrochloride*.

D. MISCELLANEOUS METHODS

Conner and Straub (256) have devised a method for the simultaneous determination of thiamine and riboflavin. The method is based on the fact that thiamine is completely absorbed on Decalso, whereas riboflavin is not. The latter is then absorbed on Supersorb. (See Fluorimetric Assay of Riboflavin.)

Many papers have appeared on the application of the thiochrome method to the assay of thiamine in various materials. The following references are a guide to some of these applications.

Vegetable and Cereal Products (269, 270, 271, 272, 273).

Simplified procedure for cereal products (274, 275).

Foods (276, 277).

The methods for the application of the thiochrome method to biological materials will be found in the section on Clinical Methods (Vol. II).

5. THE FLUORIMETRIC ASSAY OF RIBOFLAVIN

A. INTRODUCTION

In 1933 Kuhn and Wagner-Jauregg (278) showed that concentrates of vitamin B_2 fluoresce yellow-green upon activation by blue light having a wavelength of 4300-4400 Å. Karrer and Fritsche (279), and Kuhn and Morruzi (280), have shown that the fluorescence of riboflavin is considerably affected by the solvent and the pH. It shows maximum fluorescence between pH 6.0 and 7.0. Karrer and Fritsche (*loc. cit.*) have shown that maximum fluorescence is exhibited by a 0.003% solution of riboflavin at pH 7.0. Conner and Straub (281) have shown that a linear relationship between fluorescence and concentration of riboflavin exists between the limits of 0.013 γ to 0.13 γ/ml.

At high concentration, the effect of "quenching" comes into play. The fluorescence is also affected by the presence of certain anions, such as halides, cyanide, thiocyanide, sulphite, and nitrite (226). Ferrous and ferric salts have a similar quenching effect. Ellinger and Holden (226) have made important contributions to the study of this quenching effect in regard to the fluorescence of riboflavin.

Riboflavin is rapidly destroyed by ultraviolet light, and a study of the effect of light on the vitamin at various pH levels has been made by Conner and Straub (281) among others. The following table, XVIII, taken from these authors' paper (281), shows that even diffuse daylight causes extensive decomposition of riboflavin in 24 hr., especially at pHs above 2.2. In their measurements the assay samples were buffered at pH 6.0.

TABLE XVIII

Destruction of Riboflavin by Light at Various pH Values

Nature of Illumination	pH of riboflavin solution	Loss of riboflavin following different intervals[a]			
		1 day *per cent*	2 days *per cent*	5 days *per cent*	7 days *per cent*
Diffused daylight	2.2	40	96	98	—
	4.0	84	96	96	—
	6.0	82	94	98	—
	8.0	72	96	96	—
Artificial light	2.2	12	16	16	29
	4.0	15	37	60	78
	6.0	16	38	58	79
	8.0	23	37	55	65
Darkness	2.2	—	—	—	0
	4.0	—	—	—	0
	6.0	—	—	—	0
	8.0	—	—	—	0

[a] Initial content of riboflavin in each solution was approximately 50 γ.

For this reason, Conner and Straub carry out all operations for the estimation of riboflavin under controlled illumination. For this purpose they used Eastman Safety lights equipped with series OA Wrattan filters.

Various standards have been used for comparison with the intensity of fluorescence of the unknown, *e.g.*, pure riboflavin (282), potassium dichromate, fluorescein (283, 284), and uranium glass (285), have been used as such standards.

Visual methods for the determination of fluorescence have been employed, but photoelectric techniques have been almost universally adopted in more recent years. A comparison of the visual and photoelectric fluorescent methods with the biological and microbiological assays has been conducted by Emmett *et al.* (286).

They conclude, in regard to the two fluorimetric methods (see below), that "In general, with the fluorimetric measurement, it is often difficult to know how and when to heat the samples so as to obtain the optimum values. In addition, with the visual (Flouray) procedure there is apt to be a large personal error unless the operator has had much personal experience with the photoelectric procedure; there are also objections due to possible solarizing or cell fatigue. This entails a frequent careful check-up and restandardisation of the instrument before each series of runs.''

The visual method suffers from several disadvantages. (a) The human eye is not sufficiently sensitive to a difference in brightness of a ''luminous volume.'' (b) Visual methods demand the use of standard fluorescent substances for comparison. Many biological fluorescent substances are unstable and, hence, the necessity arises of using a stable substance as a standard, whose fluorescence may differ slightly in hue. The visual comparison of two intensities of different hue is extremely difficult. For these reasons, among others, the use of photoelectric cells for the measurement of fluorescence has become almost universal.

B. THE VISUAL METHOD

(1) The Method of Weissberg and Levin (284)

These authors described two methods of extracting riboflavin from dairy products.

Method.

1. Reflux a weighed sample of the solid product three times with methanol acidulated with acetic acid, in the presence of carbon dioxide and in the absence of light, washing the residue with fresh methanol between extractions. Add a volume of acetone equal to the combined methanol extracts and refrigerate overnight at −17.78° C. (0° F.) to precipitate flocculent impurities. Filter and condense filtrate *in vacuo,* adding enough water to ensure removal of the organic solvents and to leave the riboflavin in aqueous solution. Make this final solution up to a definite volume and assay. If any turbidity is present, it must be removed by filtration, or, if of fatty origin, it may be removed by ether extraction.

This method may be applied to liquid samples but no refluxing is necessary.

2. Reflux a weighed sample three times with methanol in the presence of carbon dioxide and in the absence of light. Adsorb the riboflavin from the methanol on British Fuller's earth, using approximately 10 g. of earth for each 453.6 g. (1 lb.) of original sample, assuming the potency in riboflavin to be of the order of whey powder or dried yeast. Centrifuge and wash the earth, then elute with a mixture consisting of pyridine

1, methanol 1, water 4. A second elution should also be made on the earth. Filter the combined eluates, condense *in vacuo,* and remove the colloidal Fuller's earth by adding methanol and filtering. Add water and condense the filtrate further to remove organic solvents. Then make the liquid up to volume and assay. A control should also be made by eluting the British Fuller's earth and following through as above, deducting any fluorescence value deriving from the earth itself and reagents.

These authors use a 0.001% aqueous solution of sodium fluorescein for preparing the standards, which are calibrated against a solution of synthetic riboflavin. In the table (XIX) the correlation between riboflavin and sodium fluorescein concentrations is quoted from Weissberg and Levin's paper (*loc. cit.*).

TABLE XIX

Correlation between Riboflavin and Sodium Fluorescein Concentrations

Riboflavin *g./cc.*	Sodium fluorescein *g./cc.*	0.001% Sodium fluorescein made up to 50 ml. *ml.*
0.1	0.17	0.85
0.2	0.34	1.70
0.3	0.54	2.55
0.4	0.68	3.40
0.5	0.86	4.30
0.6	1.03	5.15
0.7	1.20	6.00
0.8	1.37	6.85
0.9	1.54	7.70
1.0	1.72	8.60

The fluorescein standards are prepared to correspond to 0.1-1 γ/ml. of riboflavin. The solution of the unknown is diluted until it falls into this range and the various dilutions are multiplied by the dilution factor to obtain the riboflavin concentration.

Various dilutions of the solution of this unknown sample are made up to 50 cc., placed in French square bottles (60 cc. capacity), and compared under ultraviolet light with the fluorescein standards in similar vessels. Rubber stoppers should not be used as they may yield fluorescent impurities to the solutions under test. These authors used, as a source of exciting radiation, nine Blue glow lamps (General Electric Vapor Lamp Company, Hoboken, N. J.) mounted in a box, the top of which acted as the filter and was made of a 16.25 cm. square of Corning glass, Violet Ultra No. 586 (Corning Glass Works, Corning, N. Y.) which transmits a radiation of approximately 3650 Å.

The bottles of unknown solution and the standards are compared by placing them on the filter, and substituting standard solutions until equal intensity of fluorescences is obtained. The concentration of riboflavin is then given in γ/cc. by conc. of standard × dilution of unknown.

C. THE PHOTOELECTRIC METHOD

(1) General

The essential parts of a photoelectric fluorophotometer are (a) a light source; (b) a filter for the incident light which will transmit the exciting radiation and absorb the radiation in the region corresponding to the fluorescent light emitted; (c) a cell or cuvette for holding the solution; (d) a secondary filter for absorbing scattered radiations and transmitting the fluorescent light; (e) a photocell; and (f) an indicating system for measuring the response of the photocell.

Light Source. A high-pressure mercury arc, such as the General Electric H-3 or H-4 in a glass envelope, is usually used. Low pressure arcs in quartz (*e.g.*, the Uviarc) have also been used.

Optical Parts. These may be of glass since the usual exciting radiation is 3650 Å, which is readily transmitted by this medium. Care should be taken, however, that the glass used has a low fluorescence.

Filters. The following Corning Filter * have been used—*Primary Filter* 511 or 511 and 038 or 554. *Secondary Filter* 351 or 368.

Photocells. Either barrier layer or photoemissive types can be used.

Electrical Circuits. See reviews by: Mueller (287, 288) and Loofbourow (289).

Many modifications of the fluorimetric photoelectric assay have been described. The modifications deal with four main points. (a) Extraction. (b) Removal of interfering pigments. (c) Freeing riboflavin in the combined state in order that it may be extracted. (d) Preparation of suitable blanks.

(2) Preparation of Extracts

Various methods of preparing suitable extracts of food products and natural materials have been proposed. Hodson and Norris (282) have extracted the riboflavin with 0.25 *N* H_2SO_4, the Collaborative Committee has suggested autoclaving with 0.1 *N* H_2SO_4, whereas Conner and Straub (281) have used 0.04 *N* H_2SO_4 on a boiling water bath. Heating the sample on a steam bath with dilute phosphoric acid has been recommended by Jones and Christianson (290). Autoclaving in 0.1 *N* HCl (291) or distilled water followed by digestion with takadiastase (292) have also been used. For animal tissues proteolytic enzymes are reported to give more complete extraction of riboflavin than autolysis or autoclaving (293).

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* Numbers refer to filters of the Corning Glass Works.

(3) Removal of Interfering Pigments

Many methods have been advanced for the removal of interfering fluorescent pigments. Ferrebee (294) absorbed his extracts on heat-activated Floridin or a synthetic absorbent Supersorb (281). The riboflavin was eluted with an aqueous solution of pyridine and acetic acid and the eluate was then treated with permanganate and H_2O_2 to remove the last traces of interfering substances. This latter step was based on the original Koschara method (295).

Hodson and Norris (282) reduced the pigments with sodium hydrosulfite and stannous chloride. Sodium hydrosulfite reduces riboflavin to a non-fluorescent product which can be regenerated on oxidation with air. The interfering fluorescent substances are, unlike riboflavin, reduced irreversibly with stannous chloride. Swaminathan (296) has found this method unsatisfactory in his hands.

In some cases, traces of interfering impurities are left which cannot be removed by the means suggested above. This difficulty can be overcome (282, 292) by adding a known amount of pure riboflavin to the unknown. The interfering substances should affect the pure riboflavin standard in the same manner as the riboflavin originally present in the assay sample.

(4) Hydrolysis of Combined Riboflavin

Takadiastase has been recommended by Andrews *et al.* (292) for freeing combined riboflavin. A combination of pepsin and takadiastase has been used by McLaren *et al.* (297). Proteolytic enzymes have been reported by Snell and his collaborators (293) to give more complete extraction from animal tissues.

(5) Preparation of Blanks

Hodson and Norris (282) reduced the riboflavin in the unknown with a solution of sodium hydrosulfite making allowance for the dilution caused by the reagent, whereas Andrews *et al.* (292) used 10-20 mg. of solid $Na_2S_2O_4$ and required no correction for dilution. A benzyl alcohol solution of $Na_2S_2O_4$ has also been used (298). Some workers have taken advantage of the fact that riboflavin loses its fluorescence at pH 11.0 to obtain their blank (299, 297).

D. FLUOROMETRIC METHODS OF ASSAY

(1) The Method of Scott, Hill, Norris and Heuser (300)

In 1939, Hodson and Norris described a method of assay of more general applicability to products containing riboflavin. This method was based on the following physical and chemical properties of the vitamin. (1) The fluorescence produced when it is irradiated with blue light. (2) It is stable to mild oxidation and reduction. (3) It is transformed into a nonfluorescent substance on reduction with sodium hydrosulfite and it can be regenerated by mild oxidation with air. (4) It is not destroyed by treatment with stannous chloride. Of all the reducible interfering substances encountered by the authors only riboflavin could be oxidized back to its fluorescing form by shaking with air.

The difficulty of the stable interfering pigments can be overcome by adding known amounts of pure riboflavin to the unknown after a preliminary measurement of its fluorescence. The extent to which the interfering pigments decrease the value of the added riboflavin is then measured and, by applying a correction factor to the unknown, the amount of riboflavin in the solution can be determined.

Stable substances in the unknown sample which fluoresce in the green are eliminated by the reduction of riboflavin to its nonfluorescent state by means of sodium hydrosulfite and determining the concentration of the riboflavin by difference.

The method of Hodson and Norris (282) proved to be satisfactory for the estimation of riboflavin in materials which did not contain highly fluorescent non-riboflavin pigments. With these latter materials, however, very high results were obtained when this method was used.

Recently Scott *et al.* (300) have published a modified method which obviates the interference of the fluorescent non-riboflavin pigments. The major modification of this new method over the old, is the substitution of a permanganate oxidation step for the removal of the interfering pigments in place of the hydrosulfite reduction step of the old procedure.

The permanganate oxidation for the removal of interfering pigments was first introduced by Koschara (295). Ferrebee (294) was the first to use this step in an assay procedure.

The method of Scott *et al.* (300) has been compared to the microbiological procedure of Snell and Strong (301) with a wide variety of material and has given results in excellent agreement.

The method of Scott *et al.* (300) is quoted in detail:

Reagents.

Acid-acetone solution. 250 ml. of *N* HCl are diluted to 1 l. with acetone.

Trisodium phosphate solution. 65 g. of $Na_3PO_4.12H_2O$ are dissolved and made to 1 l. with distilled water.

Sodium hydrosulfite solution. Five g. of $Na_2S_2O_4$ are dissolved in 100 ml. of 0.2 *M* disodium phosphate solution, 1-2 g. of Celite analytical filter aid[1] are added, and the solution is placed in an ice bath. The sodium hydrosulfite is unstable and should not be used after about 4 hr.

Glacial acetic acid.

Potassium permanganate solution. A 4% solution of potassium permanganate is freshly prepared each week.

Hydrogen peroxide solution. A 3% solution is prepared by diluting a 30% solution of hydrogen peroxide 1 : 10 with distilled water.

Standard riboflavin solution. Riboflavin of the highest purity obtainable is dried to constant weight *in vacuo* at 60° C. From this, a stock solution is prepared by dissolving 50 mg. in distilled water and making up to a volume of 1 l. The solution should be preserved in a cool place and protected from the light. The addition of a few drops of glacial acetic acid will insure an acid pH and help stabilize the solution. This standard riboflavin solution is called Solution I.

Extraction Procedure. A 1-5 g. finely ground sample of the unknown is weighed into a 250 ml. red Erlenmeyer flask[2] and 100 ml. of the acid-acetone solution are added. After thorough mixing, the mixture is gently refluxed for 1 hr. The extract is cooled to room temperature, after which it is brought to pH 6.6-6.8 with trisodium phosphate solution. Sufficient water is added to make the total volume of liquid 200 ml. After standing 5 min., the mixture is filtered through No. 12 Whatman fluted filter paper. The first 10-20 ml. of filtrate is discarded. A 10-50 ml. aliquot of the filtrate is pipetted into a 200 ml. volumetric flask. The pH of this solution is adjusted to 3.7 with glacial acetic acid. 2 ml. of 4% $KMn0_4$/10 ml. of filtrate are added, mixed, and allowed to stand for 3 min. At the end of 3 min., the solution of 3% H_2O_2 is added until the color of the permanganate is changed from purple to light brown. The solution is made to volume, 1-2 g. of Celite analytical filter aid are added, and the solution is filtered through No. 44 Whatman filter paper. The first 10-20 ml. of filtrate are discarded, in order to eliminate turbidity in the filtrate caused by a few small particles which sometimes pass through the filter paper before the filter aid has formed an efficient mat.

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[1] Obtained from Johns-Manville.

[2] Pyrex Brand Lifetime Red glassware obtained from the Corning Glass Works, Corning, N. Y.

A known volume of this clear solution is then pipetted into the optical cell for the fluorometric determination.

Twenty ml. of Solution I were dissolved in 100 ml. of acid-acetone solution neutralized to pH 6.6-6.8 with trisodium phosphate solution and made to a volume of 200 ml. with water. Forty ml. of this solution are taken to pH 3.7 with glacial acetic acid, treated with 8 ml. of $KMnO_4$ solution for 3 min., almost decolorized with 3% H_2O_2, made to a volume of 200 ml., and filtered through a No. 44 Whatman filter paper after addition of Celite filter aid. This solution is used as the standard in determining the riboflavin content of the unknowns. This standard riboflavin solution is called Solution II. The pH of this solution should be identical with that of the unknowns. If carried out as above, it will be approximately pH 4.0. This solution may be kept in a red flask in the refrigerator for at least a week.

Fluorometric Determination. A distilled water blank is placed in the fluorophotometer and the galvanometer is set at zero. One ml. of the Solution II (1 γ/ml.) is added to 15 ml. of distilled water and this reading is noted. This is called reading *S*. Then 15 ml. of the unknown solution are pipetted into the glass cell and a reading (*A*) is made. The glass cell is removed from the path of light and 1 ml. of the Solution II is added and a second reading (*B*) made. After this the riboflavin in the solution is reduced to the non-fluorescing leuco form by adding 1 ml. of the sodium hydrosulfite solution and a third reading (*C*) is made. Reading *S* is taken again to make sure that the light intensity has not changed during the above operations.

Calculation of Results. The γ of riboflavin/ml. of unknown solution are calculated as follows:

(1) $\frac{A\text{-}C}{B\text{-}A} D = \gamma$ riboflavin/ml. unknown solution (uncorrected) where $A =$ fluorescence reading of 15 ml. aliquot of unknown solution; $B =$ fluorescence reading of unknown solution after addition of 1 ml. of standard riboflavin Solution II (total volume 16 ml.); $C =$ fluorescence reading after further addition of 1 ml. of sodium hydrosulfite solution (total volume 17 ml.); $D = \gamma$ of added riboflavin/ml. of solution at the time reading *B* is taken ($D = 1\ \gamma/16$ ml. $= 0.0625\ \gamma$/ml.).

However, since reading *A* was taken in 15 ml., while reading *B* was taken after dilution of the solution to 16 ml., and reading *C* was taken when the total volume of the solution was 17 ml., readings *B* and *C* must be corrected to the values that they would have been if the readings had been made in 15 ml. of solution in each case. This is accomplished by the use of correction factors *y* and *z* ($y = 16/15 = 1.067$ and $z = 17/15 = 1.133$) as shown in Eq. 2.

(2) $\frac{A\text{-}Cz}{By\text{-}A} D = \gamma$ riboflavin/ml. unknown solution (corrected)

Since Eq. 2 gives the γ of riboflavin/ml. of unknown solution, the amount of riboflavin/g. of sample is obtained by multiplying the result of Eq. 2 by the dilution factor f ($f = 400$ if the original extract of a 5 g. sample is diluted to 200 ml. and a 20 ml. aliquot of this is further diluted to 200 ml.). Thus, the final equation, which is used in determining the γ of riboflavin/g. of sample is,

(3) $\frac{A\text{-}Cz}{By\text{-}A} Df = \gamma$ riboflavin/g. unknown.

(2) The Method of Jones and Christianson

Jones and Christianson (290) introduced several modifications into the Hodson and Norris (282) procedure. The main difference lies in the extraction of the riboflavin from the sample by means of dilute phosphoric acid. Their method is quoted below:

a. Instrument. We have used the Pfaltz and Bauer Fluorophotometer, Model A (a newer type is now available) with a G. E. Mercury Lamp, Type H3, 85 watts, as the source of light. Filter glass (Jena) combination GG-15 and BG-12, giving a peak at 4500 Å., is placed between the the solution in the 4 cm. glass cuvette and the source of light. An orange filter glass 315, Corning, is placed between the cuvette and photocell. The iris diaphragm, controlling the amount of light admitted to the solution in the cuvette, is opened about 3 cm.

The intensity of the activating light is regulated to give a definite galvanometer reading. This may be done in two ways: first, by using a standard solution of quinine activated by ultraviolet light at 3650 Å. (this requires switching of filter glasses during testing period), or, second, by direct galvanometric setting for the transmitted light picked up by the rear photocell. Once the resistance has been set for a definite fluorometric reading, the intensity of light is held constant by opening or closing the iris diaphragm, with a voltage stabilizer in the line. This change in intensity of light is very small.

b. Reagents. Standard Riboflavin Solution A. Fifty γ of anhydrous riboflavin are dissolved in 500 cc. of 25% aqueous alcohol containing 2.0 cc. of 85% phosphoric acid. The solution is kept in an amber bottle in the refrigerator. Each cc. contains 100 γ riboflavin.

Standard Riboflavin Solution B. Prepared daily by diluting 5 cc. of standard solution and 2.0 cc. of 85% phosphoric acid to 500 cc. with distilled water.

N/10 Sodium hydroxide.

Sodium hydrosulfite solution: One g. of sodium hydrosulfite and 1

g. of sodium bicarbonate are dissolved in 20 cc. of ice-cold distilled water and kept in an ice bath.

c. Procedure. To the sample (1-2 g. of powders, 1-2 cc. of liquids or 2 capsules) in a 250 cc. Erlenmeyer flask, are added 100 cc. of distilled water and 0.4 cc. of 85% phosphoric acid solution. The flask is shielded from light by means of close fitting blackened tin. The mixture is heated on the steam bath for one hr., cooled, and filtered by means of paper into a volumetric flask. Any insoluble matter on the filter is washed with distilled water. The filtrate is made up to such a volume that each cc. will contain about 1 γ of riboflavin; 0.4 cc. of 85% phosphoric acid is added for each 100 cc. additional volume over and above the original 100 cc. of water added. The measurement of fluorescence is carried out as follows, having the instrument regulated as described above:

(*a*) To 2 cc. of the solution on test, in a 25 cc. amber cylinder, are added 2 cc. of the $N/10$ NaOH and distilled water to make 25 cc.; 12.5 cc. of this solution are placed in the 4 cm. cuvette and a galvanometer reading is recorded.

(*b*) Two cc. of the Standard Riboflavin Solution B are treated as in (*a*).

(*c*) To a second 2 cc. portion of the solution on test in a 25 cc. amber cylinder are added 2 cc. of Standard Riboflavin Solution B, 4 cc. $N/10$ NaOH and sufficient distilled water to make 25 cc. Measurement carried out as under (*a*).

(*d*) Repeat (*c*) except that 2 cc. of the hydrosulfite solution are added prior to making up to 25 cc. with distilled water. This constitutes the blank.

(*e*) To 2 ml. of the Standard Riboflavin Solution B are added 2 cc. of $N/10$ NaOH solution, 2 cc. of the hydrosulfite solution and sufficient distilled water to make 25 cc. Then 12.5 ml. of this solution are placed in the cuvette and the fluorescence reading on the galvanometer is recorded. This constitutes a blank, which varies from 1.5-3.5. The difference between (*b*) and (*e*) equals the reading for 1 γ of riboflavin.

$$\gamma \text{ riboflavin} = \frac{a-d}{b-e} \times \frac{b}{c-a} \times \text{dilution} \times \frac{1}{\text{amt. of sample}}$$

A comparison between the Hodson-Norris method and the above method showed fair agreement between the two methods.

(3) The Method of Conner and Straub (281)

These authors make use of the fact that Supersorb, introduced by Ferrebee (294), will absorb riboflavin completely from acid solution, while Decalso does not, although the latter completely absorbs thiamine from aqueous solutions containing riboflavin. On the basis of these observations, these authors developed a method for the simultaneous determination of thiamine and riboflavin (256).

Since riboflavin is sensitive to light, these authors carried out the entire analysis under controlled illumination. For this purpose, they used Eastman safety lamps equipped with series OA Wrattan light filters.

The preparation of samples for the combined determination of thiamine and riboflavin is described by Conner and Straub (256). The material should be in a finely pulverized form, which is attained in the case of fresh vegetables, for example, by freezing them with solid carbon dioxide and then grinding them in an ordinary meat grinder and weighing a representative sample of the vegetable. These authors recommend that the grinding and weighing should be carried out in a refrigerated room held below 0° C.

A preliminary ether extraction in a Soxhlet extractor is necessary when the sample is high in fat content.

The weighed sample (3-5 g.) is placed in a tubular extraction vessel of about 75 cc. capacity attached to a small reflux condenser by means of a ground glass joint. Through the condenser passes an electrically driven stirrer whose speed can be varied by means of a rheostat.

The sample is extracted with 50 cc. of 0.4 *N* H_2SO_4 on a boiling water bath for approximately 1 hr., with continuous stirring. At the conclusion of the extraction, each stirrer is carefully washed down with 5 cc. of distilled water delivered from a graduated pipet and the tube is cooled to room temperature with running water. Ten cc. of a 5% solution of clarase (256) are added to each tube and the contents are thoroughly mixed with a glass stirring rod. The tube, containing the stirring rod, is then incubated at 45° C. for 2 hr. Following the incubation period, each stirring rod is carefully washed down with exactly 1 cc. of distilled water and the extraction tube is cooled to room temperature. Each tube is then centrifuged at high speed until a clear supernatant liquid is obtained.

The aliquot to be taken from the supernatant liquid will be dependent upon the amounts of thiamine and riboflavin present in the extract. A single aliquot may be used if it contains about 5 γ of thiamine and 1 γ of riboflavin. Otherwise, separate aliquots should be taken for the determination of each vitamin. The adsorption apparatus used for the combined determination of riboflavin and thiamine consists of two glass adsorption columns (256), connected by a No. 6½ two-hole rubber stopper so that the filtrate from the upper column falls directly onto the lower column. The stopper is provided with a small one-way glass stopcock to regulate the flow of liquid through the upper column. The combined columns are attached to a modified vacuum receiver (Scientific Glass Apparatus Co., Bloomfield, N. J.) connected to the laboratory suction outlet.

The upper column is filled with activated 60- to 80-mesh Decalso, and the lower column with 60- to 80-mesh Supersorb prepared as described by Ferrebee (294). In the original method, the adsorption of riboflavin was carried out with a column 15 cm. long and 1 cm. in diameter filled with 30- to 60-mesh Supersorb. However, it was found that if 60- to 80-mesh Supersorb were used, a column of design similar to that used for the adsorption of thiamine could be employed (256).

The solution containing the riboflavin and thiamine is allowed to pass through the two columns at the rate of 1 cc./min., the flow being regulated by adjusting the stopcock between the two columns as well as that on the vacumm receiver. (The stopcock on the vacuum receiver is lubricated with glycerol or orthophosphoric acid to avoid fluorescent impurities present in other lubricants.) Thiamine is adsorbed on the Decalso, but the riboflavin passes through it and is adsorbed on the lower column of Supersorb. Both columns are washed down thoroughly with hot distilled water and the washings are discarded. The upper column containing the Decalso is then detached and transferred to a 125 cc. glass suction flask where it is ready for elution of the thiamine with 25% KCl (256).

The lower column containing the Supersorb is detached from the vacuum receiver, which is washed with hot water and dried by pulling air through it. The column is then attached to the receiver again and the riboflavin eluted with 25 cc. of a solution of 20% pyridine in 2% acetic acid, added from an automatic pipet, the flow through the column being adjusted to 1 cc./min. (The pyridine solution should have a low fluorescence and be stored in a dark bottle.) Additional small portions of the eluant are then passed through the column until the volume of the filtrate is approximately 50 cc. as indicated by the graduated readings on the vacuum receiver. In analyzing most food materials, the filtrate is then made up to exactly 50 cc. with the pyridine-acetic acid mixture. However, for concentrates of riboflavin it may be necessary to make up the filtrate to a larger volume, so that a 15 cc. aliquot will contain from 0.5 to 1.0 γ of the vitamin.

A 15 cc. aliquot of the riboflavin filtrate is pipetted into a 50 cc. brown bottle or flask, 1 cc. of a 4% solution of $KMnO_4$ is added and, after an interval of a minute, 3 cc. of a 3% solution of H_2O_2. The latter can be conveniently prepared by diluting a 30% solution of H_2O_2 (Superoxol) a few minutes before the determination is to be run. The $KMnO_4$ solution should be prepared fresh daily. The reaction mixture is shaken vigorously and then allowed to stand until all effervescence has ceased, as the presence of bubbles will introduce an error in the fluorescence readings. Fifteen cc. of the solution are pipetted into a cuvette of the fluorophotometer, and the fluorescence readings are made after adjusting the instrument (256). A Corning glass filter No. 511 is used for transmitting the incident light and No. 351 for the fluorescent light. Both filters are of 2 mm. thickness.

Although in the range of 0.2-2.0 γ a linear relationship exists between the amount of riboflavin and galvanometer readings, it has been found advisable to make a daily determination on a standard solution of riboflavin in pyridine-acetic acid instead of depending entirely upon a calibration curve. The standard solution is made up to contain 0.0856 γ of riboflavin/cc. and should be kept in a dark colored bottle away from light. No destruction of the vitamin occurs under these conditions.

For determining the fluorescence of the standard solution, a 15 cc. aliquot is treated with 1 cc. of the 4% solution of $KMnO_4$ and 3 cc. of 3% H_2O_2. Fifteen cc. of this solution, representing 1 γ of the vitamin, are then pipetted into the cuvette of the fluorophotometer and its fluorescence is determined. The standard solution must be treated in the same manner as the sample, since the fluorescence of riboflavin solutions is dependent upon the solvent employed as well as upon the hydrogen ion concentration. A blank correction for the standard solution is obtained by determining the fluorescence of 15 cc. of a solution taken from a mixture of 1 cc. of 4% $KMnO_4$, 3 cc. of 3% H_2O_2, and 15 cc. of the pyridine-acetic acid reagent.

A "complete blank" (256), consisting of all the reagents used throughout the method, is also run through the entire procedure and serves as a blank for the thiamine determination as well as for the riboflavin. In the case of riboflavin the value for the blank is subtracted from fluorescence readings of the sample.

Conner and Straub showed that the results obtained by this method were in good agreement with the biological rat growth method for the assay of riboflavin. The difference in results by the two methods were well within the range of error inherent in the rat growth method.

Andrews (302) has pointed out that this method gives good results with a variety of products, but, in the case of bread, there are indications that the removal of non-flavin pigments is incomplete, thus introducing appreciable errors.

(4) The Method of McLaren, Cover, and Pearson (297)

These authors make use of the observation that the fluorescence of riboflavin is destroyed at pH 11.0. They claim that their method gives results in the assay of meat products in good agreement with the microbiological method of Snell and Strong.

Method.

Extraction of riboflavin from meat is accomplished with a combination of enzymes, papain and takadiastase at pH 4. Impurities in the resulting extract appear to be mainly fat and protein. Fat is removed effectively with chloroform. Most of the remaining protein material is removed by precipitation at pH 6.8-7.0.

Fluorimetric measurements are made on the unknown at pH 6.8-7.0, at which riboflavin exhibits maximum fluorescence. The necessity of removing traces of interfering pigments is overcome by adding a known amount of the standard riboflavin to the unknown. A blank then is obtained by adding one drop of saturated solution of NaOH to bring the solution to pH 11 at which the fluorescence of riboflavin is destroyed.

It is necessary to carry out the entire analysis under subdued light.

Preparation of Extract. A 4 g. sample of finely ground meat is weighed into a 250 ml. flask.[1] To the flask is added 100 ml. of 0.1 *M* sodium acetate buffer (pH 4), 0.1 g. each papain (Difco) and takadiastase, and 4 drops concentrated HCl. The contents of the flask are stirred at 300 r.p.m. for 2 hr. A few drops of benzene are added and the flasks placed in an oven (45-50° C.) for approximately 15 hr.

Removal of Interfering Substances. Three ml. of chloroform are added, the flasks shaken vigorously for 2 min. and then allowed to stand until the chloroform settles to the bottom. The digest is decanted and filtered first through filter paper[2] and then through a Hirsch sintered glass funnel covered with a very thin layer of Filter-Cel. An aliquot of 50 ml. is adjusted to pH 6.8-7.0 with NaOH, and allowed to stand overnight in the refrigerator. It is filtered through filter paper[2] and the precipitate washed several times with distilled water. The volume of filtrate and washings is made up to 150 ml.

Fluorometric Readings. The photofluorometer used in this laboratory is a Coleman Model 12, fitted with filters B-2 and PC-2. A compensating transformer is used to deliver constant voltage to the mercury lamp. The instrument is standardized with a fluorescein solution (0.1 mg./ml. of water) to give a deflection of 30-40 scale divisions.

A 15 ml. aliquot of extract is pipetted into a cuvette and the fluorimetric reading (*A*) taken. One ml. of standard riboflavin solution containing 1 mg./ml. is added. After agitation, the reading (*B*) is taken. One drop of saturated NaOH is added, which brings the solution to approximately pH 11 and destroys the riboflavin. After agitation, the reading of the blank (C) is taken. The riboflavin content of the sample is calculated from the equation:

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[1] Centrifuge bottles of 250 ml. capacity were used.

[2] Whatman No. 4 filter paper was used.

$$\left(\frac{A - 1.06\ C}{B - 0.94\ A} \times 0.063 \times \frac{300}{\text{Wt. of sample}}\right) - \frac{\text{Enzyme}}{\text{blank}} = \gamma/\text{g}.$$

where A, B, and C are the instrument readings in scale divisions, the numerical values 1.06 and 0.94 are correction factors which compensate for dilution with the added solution of pure riboflavin, 0.063 is a contraction of

$$\left(\frac{\text{Vol. at } A}{\text{Vol. at } B} \times \frac{\gamma \text{ riboflavin in standard}}{\text{Vol. of aliquot}}\right)$$

and 300 is a contraction for the original volume of the extract (100) times the dilution factor $\left(\frac{150}{50}\right)$.

(5) *The Methods of the Riboflavin Assay Committee*

In 1943, Andrews (302) reported on the fluorimetric method of assaying riboflavin which had been set up by the Research Corporation Collaborative Committee on the Riboflavin Assay.

The method proposed was devised to minimize the adverse effects of cereal pigments which are present in bread due to carmelization, *etc.* The two alternative methods are based largely on those of Conner and Straub (281) (absorption on Florisil; synonymous with Supersorb (294) followed by permanganate treatment) and the compensatory method of Hodson and Norris (282) (measurement of extracts before and after addition of a known amount of riboflavin).

The method used is quoted as follows:

A 5 g. sample of the bread or whole wheat flour is autoclaved at 15 lbs. pressure for 15 min. with 50 ml. of 0.1 N H_2SO_4. After cooling to about 50° C., the suspension is quickly neutralized to a pH of approximately 6 with a 6.5% $Na_3PO_4.12H_2O$ solution (this requires approximately 12 ml.). One ml. of freshly prepared 6% takadiastase solution is then added and allowed to stand for ¾ hr. in the dark. After making up to a total volume of 100 ml., the mixture is filtered, the first 10-15 ml. of filtrate being discarded.

A 20 ml. aliquot of the filtrate is passed through a column (see Note 1) of Florisil. After the 20 ml. portion has completely passed through the column, the effluent is discarded and the column washed with 5-10 ml. of hot distilled water. It is then dried by drawing air through the capillary end of the tube. The absorbed riboflavin is then eluted with 15 ml. of 20% pyridine in 2% acetic acid (see Note 2), the eluate being collected in a 25 ml. glass stoppered graduate. It is then made up to a total volume of 20 ml. with the pyridine-acetic acid solution and thoroughly mixed. If cloudy, this must be filtered before making fluorimetric measurements.

A 14 ml. aliquot of this eluate is pipetted into the cuvette of the fluorophotometer (see Note 3), and the fluorescence (*A*) determined. Prior to this measurement, the light intensity of the fluorophotometer is standardized by means of a solution of sodium fluorescein (see Note 4). The concentration of this fluorescein solution will depend upon the purity of the sodium salt. A considerable variation has been found in commercial products. Concentration should be such that its fluorescence will be approximately the same as that of a solution containing 0.2 γ/ml. of pure riboflavin and will range somewhere between 0.025 and 0.1 γ/ml. Adjustment of the light intensity is made by means of the iris to give a galvanometer deflection of 25 divisions (see Note 5). Light filters are 351 for the fluorescent light and a 511-038 combination for the incident light.

Following the determination of the fluorescence of the eluate (*A*), 1 ml. of solution containing 1.5 γ (see Note 6) of pure riboflavin in 20% pyridine and 2% acetic acid is added to the contents of the cuvette with stirring and the resultant fluorescence (*B*) determined. A few crystals of solid sodium hydrosulfite (10-20 mg.) are added and the solution stirred until the riboflavin color has been destroyed (or the hydrosulfite dissolves). The fluorescence (*C*) is then recorded.

The riboflavin content of the sample is calculated by means of the following equation:

$$\frac{A - 1.07C}{B - 0.93A} \times 0.1 \times \frac{100 \text{ ml.}}{5 \text{ g.}}$$

where the values 1.07 and 0.93 are dilution factors (see Note 6): *A, B,* and *C* are the galvanometer deflections in scale divisions; and 0.1, the concentration of the added riboflavin/ml.

Notes.

1. The tubes containing this Florisil have an inside diameter of 6 mm., fitted at the lower end with a capillary, which, when the column contains approximately 1 g. of Florisil, gives a rate of flow through the column of about 1 ml./min. The top of the tube is belled to contain 15-20 ml. of solution. The height of the actual Florisil layer is approximately 2.5 in.

2. Mallinckrodt's "Analytical Reagent" quality pyridine has been found to be satisfactory without further purification. However, each

collaborator should check his pyridine for fluorescence and, if it is appreciable, should purify by distillation.

3. This quantity is recommended where the Pfaltz and Bauer instrument is employed. Other instruments may require other quantities, depending upon the capacity of the cuvette.

4. Sodium fluorescein solution, because of its greater light stability, is preferred to riboflavin solutions for standardizing the light intensity.

5. Once again, this is based on the Pfaltz and Bauer instrument.

6. Where cuvette capacities require other than 14 ml. of the eluate, this concentration should be adjusted to add an amount of riboflavin equivalent to 0.1 γ/ml. of total solution. Similar volume corrections will be required in the "dilution factors" used for calculating the final results ($15/14 = 1.07$, $14/15 = 0.93$).

An alternative method was proposed in the same paper as follows:

A 5-10 g. sample of bread is suspended in 50 ml. of 0.1 *N* H_2SO_4 and autoclaved for 15 min. at 15 lb. pressure. While still hot, 12 ml. of 6.5% $Na_3PO_4.12H_2O$ is added, and, when the temperature reaches about 50° C., 1 ml. of 6% takadiastase is stirred in. After standing about ¾ hr. the suspension is made up to 100 ml. and filtered, the first 10-15 ml. of filtrate being discarded. To a 25 ml. aliquot of the filtrate is added 0.5 ml. of glacial acetic acid and 1 ml. of 4% $KMnO_4$ solution. After 1 min., 2 ml. of 3% H_2O_2 is added to decolorize the excess permanganate. The total solution (28.5 ml.) is passed through the column of Florisil and, after washing and drying, the riboflavin is eluted with about 20 ml. of 20% pyridine in 2% acetic acid. The eluate is made up to 25 ml. with the pyridine-acetic acid, and a 14 ml. aliquot is taken for the fluorescence examination previously described.

The results of this collaborative study, which was combined with a study of the microbiological assay method, demonstrated that these types of assays yield comparable average results when applied to whole wheat flour, whole wheat bread, and dried yeast. The results of individual investigators however, showed wide variations.

A second collaborative study was carried out with main emphasis on the fluorimetric method (303). In this study the emphasis was laid on two factors, (a) the use of acid extraction to liberate combined forms of riboflavin and (b) the size of the sample. The method is given in reference (303).

In a third collaborative study (304), emphasis was placed mainly on the use of permanganate and Florisil in the fluorimetric assay when applied separately to the purification of cereal extracts. An untreated extract was compared with an extract treated with Florisil and another treated with permanganate.

The results showed that, in the case of enriched flour, neither the Florisil nor the permanganate treatment is necessary, as direct reading of the untreated extracts gave satisfactory results.

In the case of enriched bread, the permanganate treatment removed considerable interfering impurities. Complete removal of the impurities, however, was not achieved. Florisil had no effect on the extracts of enriched bread.

It has been pointed out by Andrews, in a paper presented to the Minnesota Institute of Cereal Chemists (305), that the assay values are lowered as the quantity of sample for analysis is increased. Hoffer, Alcock and Geddes (306) have indicated that this anomaly may be due to two factors (1) adsorption of riboflavin on residues remaining after extraction; (2) incomplete adsorption by Florisil at higher concentrations of riboflavin. They also pointed out that the native riboflavin of flour extracts is less readily adsorbed by Florisil than pure riboflavin. Native riboflavin absorbed on Florisil is removed by washing with water and a minimum amount of wash water should be used. These authors recommend the use of a combination of Corning filters 511 and 038 for the incident light and 351 for the fluorescent light.

(6) Miscellaneous Methods

In the assay of high potency products for riboflavin, the fluorimetric assay has been shown to give results in good agreement with the biological assay (332, 282, 160, 286, 290, 307, 308).

It has been pointed out (297, 302, 303, 307, 309) that serious divergence of assay values is found when the usual methods are used for low potency products.

For such low potency products, Rubin *et al.* (310) have proposed a modification of the Hodson and Norris method (282). With this method the authors obtained excellent agreement with the results of the microbiological assay and a very considerable saving in time. The method of these authors is quoted below:

Reagents.

Standard Riboflavin Solution. Stock. Dissolve 75 mg. of pure crystalline riboflavin in 0.05 N H_2SO_4 to make 1000 ml. of solution. Prepare monthly (1 ml. contains 75 γ). *Working.* Dilute 20 ml. of stock solution to 100 ml. with distilled water. Prepare daily (1 ml. contains 15 γ).

Stannous Chloride Solutions. Stock. Dissolve 10 g. of $SnCl_2$ in 25 ml. of conc. HCl and store in a dark glass bottle at room temperature. *Working.* Dilute 1 ml. of stock solution to 500 ml. with distilled water. Prepare daily.

Sodium Hydrosulfite. (2.5%). Dissolve 1 g. in 40 ml. of 2% $NaHCO_3$ solution. Stable in ice bath for 2-4 hr.

Sodium Acetate Solution. (2.5 *M*). Dissolve 4 g. of $KC_2H_3O_2.3H_2O$ in distilled water to make 1 l. of solution.

Permanganate Solution. (4%). Dissolve 4 g. of $KMnO_4$ in distilled water to make 100 ml. Prepare every two weeks.

Hydrogen Peroxide Solution. (3%). Dilute 5 ml. of 30% H_2O_2 to 50 ml. with distilled water. Store in refrigerator and prepare every two weeks.

Clarase (Takamine Laboratories, Clifton, N. J.). It is desirable to have a preparation of low riboflavin content, less than 5 γ/g. All lots must be assayed.

Sulfuric Acid, approximately 0.1 *N*.

Apparatus. A fluorophotometer (1) exhibiting sufficient sensitivity to the fluorescence of riboflavin, (2) yielding a linear relationship between galvanometer readings and critical riboflavin concentrations, and (3) demonstrating stability of response during operational period. For general practicability it is desirable to operate the instrument so as to obtain a galvanometer response of 40-50 scale divisions for riboflavin concentration of 0.1 γ/ml. The Pfaltz and Bauer fluorophotometer, Model B, the Coleman Universal spectrophotometer, Model 12, the Coleman Universal spectrophotometer, Model 11, with UV lamp and 20×40 mm. cuvettes, and others are satisfactory.

A Waring Blendor or Wonder-Mix.

A shaking machine with vigorous to-and-fro action.

An autoclave or water bath.

An incubator or low-temperature oven.

Light-protecting equipment, such as red or amber glassware, Eastman safety lights with series OA Wratten light filters. All solutions containing riboflavin should be protected from the light.

Preparation of Extract for Fluorimetry. All samples may be acid-extracted, incubated with clarase, and permanganate-treated (with recovery step). When in doubt about the value of the clarase or permanganate step, include these in the procedure. It is desirable to use clarase on all products of a starchy nature and on animal tissue. Permanganate treatment should be used on all extracts which are appreciably pigmented.

Acid Extraction. Weigh a quantity of sample (not more than 20 g. of dry material) preferably containing about 35 γ of riboflavin, and mix with 150 ml. of 0.1 *N* H_2SO_4. If the sample is not finely pulverized, mix in a Blendor and transfer the contents to a flask easily accommodating a volume of 250 ml. If the sample is finely pulverized, introduce the dry product into the flask, add the acid solution, and mix by hand-shaking. Either immerse the flask in a boiling water bath or reflux for 45 min., and swirl frequently, or autoclave at 6.8 kg. (15 lb.) pressure for 15-20 min. Cool and add 10 ml. of 2.5 *M* $NaC_2H_3O_2$ solution. If the sample clumps during heating, again macerate in the Blendor. Dilute to 250 ml. with distilled water and filter through Whatman No. 40 paper.

Enzyme Treatment. If the sample is to undergo enzyme digestion, omit the above dilution and filtration steps, add 3% of the sample weight of clarase (dispersed in the sodium acetate solution), and conduct the digestion for 2 hr. at 45-50° C. Cool, dilute to 250 ml. and filter. (This filtrate can also be used for thiamine assay by the methods of Hennessy (262) or Conner and Straub (256, 281)).

Permanganate Oxidation. Measure 50 ml. of the filtered extract, obtained either after acid extraction or enzyme treatment, into each of two 125 ml. clear-glass Erlenmeyer flasks, U and R. To one, R, add 0.5 ml. of riboflavin standard working solution (for recovery purposes). To each flask add the same amount of 4% $KMnO_4$ solution (2 ml. or more), swirl, and allow to react for 2 min. Then add dropwise (titrate) with swirling, just enough 3% H_2O_2 solution to cause a disappearance of the permanganate color. This may or may not be accompanied by formation of a precipitate. If a precipitate forms, add H_2O_2 only as long as it continues to react, as indicated by progressive diminution of a color due to solution of the precipitate and the fizzing sound caused by evolution of oxygen. Since the reaction is not instantaneous, add H_2O_2 carefully, with swirling. If the color is disappearing, stop the addition of H_2O_2 when a slight color remains, which will disappear completely after several minutes. If an excess of H_2O_2 is suspected, back-titrate dropwise with 4% $KMnO_4$ until the color of the added permanganate fades slowly. Transfer quantitatively to a 100 ml. glass-stoppered graduated cylinder, add a drop of caprylic alcohol, and dilute to 70 ml. Filter, if necessary, through Whatman No. 42 paper.

For occasional samples, such as some mixed feeds, filtration at pH 4.5 is very slow and may yield cloudy, colored filtrates. In such cases, add 1 *N* sodium phosphate solution, after the hydrogen peroxide treatment, until the precipitate becomes flocculent, dilute to 70 ml., and filter through Whatman No. 42 paper.

Fluorimetry. In the preparation of extracts for fluorimetry a sufficient volume of solution is prepared, so that a duplicate set of readings may be taken on a given sample.

With Permanganate Treatment. Measure 50 ml. of solutions U and R obtained after permanganate treatment into sulfite solution and 2.5 ml. of the working stannous chloride solution, allow to stand 10 min., and then shake vigorously (unstoppered) in the shaking machine for 15 min. From solution U, pipet 15 ml. into the cuvette and take galvanometer reading A with the standardized fluorophotometer. (Use 15 ml. for Coleman Model 12 and Pfaltz and Bauer Model B; use 30 ml. for Coleman Model 11.) Protect the cuvette from the incident light beam of the instrument, add 0.1 ml. of the riboflavin standard working solution (the addition of 0.1 γ of riboflavin/ml. of solution), and mix thoroughly with a small glass rod. (Use a micropipet or serological pipet of 0.1 ml. capacity to add the riboflavin solution. Wipe outside of pipet and "tip to the mark." In delivering the contents of the pipet, place the tip just above the meniscus at the side of the cuvette, allow to drain, then gently blow clean.) Take galvanometer reading B. Then add one drop of the sodium hydrosulfite solution, stir, and take blank reading C. Add a second drop of sodium hydrosulfite solution to check the completeness of riboflavin reduction. If there is no significant change in the reading upon the addition of a second drop, reduction was complete after the first addition.

From solution R, take reading R_1 of a 15 ml. quantity in the cuvette, then reduce the riboflavin by dropwise addition of the sodium hydrosulfite solution and take blank reading R_2.

Without Permanganate Treatment. Measure 50 ml. of the filtrate obtained after acid extraction or enzyme treatment into a 100 ml. graduated cylinder, add 1.0 ml. of the sodium hydrosulfite solution and 2.5 ml. of the stannous chloride working solution, dilute to 75 ml., and allow to stand for 10 min. Then pour 45-50 ml. into an unstoppered 500 ml. Erlenmeyer flask and shake vigorously for 15 min. Take fluorescence readings A, B, and C as described for solution U.

Calculations:

W = weight of sample
S = γ of standard riboflavin added/ml. of solution = 0.1
V = total ml. of dilution of W = 375
E = riboflavin content of clarase in γ/g.
0.03 E = clarase blank in γ/g. of sample.

$$\frac{A-C}{B-A} \times S = \gamma \text{ of riboflavin/ml. of sample extract.} \quad (1)$$

$$\left(\frac{A-C}{B-A} \times S \times \frac{V}{W}\right) - 0.03\, E = \gamma \text{ of riboflavin/g. of sample.} \quad (2)$$

The use of the micropipet or serological pipet eliminates the need for volume correction factors for readings B and C. The same amount of riboflavin may be added in a larger volume if the proper volume corrections are applied.

If permanganate treatment is employed, the following index can be used to evaluate the recovery of riboflavin during the permanganate treatment step.

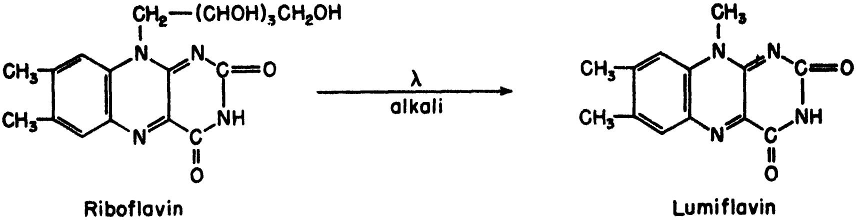
$$\frac{R_1 - R_2}{B - C} \times 100 = \text{permanganate recovery index} \quad (3)$$

A value of approximately 100% indicates no destruction of riboflavin. If the value is less than 90%, riboflavin destruction during permanganate treatment may be suspected, and the assay should be repeated.

Several modifications of this method have been suggested by Loy (311).

(7) The Lumiflavin Method

When riboflavin is irradiated in alkaline solution it is converted into lumiflavin.



Lumiflavin is soluble in chloroform and can thus be extracted from the irradiation solution, after acidification, and then determined by its fluorescent absorption spectra (312, 313, 314). The conversion of riboflavin into lumiflavin is not quantitative and thus a direct method has not been worked out. However, by irradiating a solution of pure riboflavin of the same concentration as the unknown and under the same conditions (pH, temperature, and amount of air present) and determining the lumiflavin formed, a correction factor can be worked out. This assay can be made still more exact by adding a further correction factor which can be obtained by determining the recovery of a known amount of pure riboflavin added to the unknown before irradiation.

This latter factor takes care of the interfering chloroform-soluble substances produced during the irradiation. By correlating these three figures a fairly accurate determination of riboflavin may be made.

6. THE FLUORIMETRIC ASSAY OF 4-PYRIDOXIC ACID (β-PYRACIN) *

Huff and Perlzweig (315) have isolated 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (II) (4-pyridoxic acid) from urine after the ingestion of pyridoxine (I). This acid and its corresponding lactone (β-pyracin (III)) fluoresce (the latter more intensely than the former), and a method of assay based on this property has been worked out (315).

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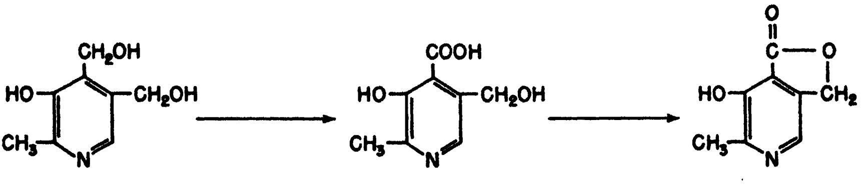
* This name was proposed for the lactone by Norris *et al.* (316).



I II III

The method is quoted from the article by the above authors:

The fluorescence measurements were made in the Coleman Model 12 electronic photofluorometer, equipped with Filters B1 and PC1, employed for thiochrome determinations; the galvanometer was set at 100 with a solution containing 0.3 γ of quinine sulfate/ml. of 0.1 *N* H_2SO_4. The aqueous solutions were diluted to bring them within the range of the instrument and the reaction adjusted to pH 3 to 4 for the metabolite (II) usually with glacial acetic acid, and for the lactone (III) to pH 9 ± 0.3 by the addition of solid sodium borate. Since the lactone form (III) gives a fluorescence intensity 25 times that of the metabolite (II), the latter was determined preferably as the lactone, after being heated is to heat 1.00 ml. of urine with 9 ml. of 1 : 10 solution of concentrated HCl in a covered test-tube by immersion in boiling water for 15 min. After cooling, the urine is diluted to a known volume in order to bring the reading within the range of the instrument. The solution in the cuvette is brought to pH 9 by the addition of solid sodium borate. The blank reading is obtained from a sample of unheated urine in the same dilution and at the same pH. With the instrument used in this study, a convenient standard consisted of a solution containing 0.04 γ of the lactone/ml. of about 1% sodium borate solution. The standard is not stable at this pH and is made up as required from a stock solution containing 0.4 γ/ml. in 0.1 *N* HCl solution.

7. THE FLUORIMETRIC ASSAY OF THE METABOLIC PRODUCTS OF NICOTINAMIDE

Najjar and his colleagues (317, 318, 319, 320) have shown that there is a fluorescent pigment (F_2) normally present in the urine of dogs and man. On ingestion of nicotinamide and related compounds, the elimination of this compound increased, whereas the urine of pellagrins was devoid of F_2. Coulson *et al.* (321) developed a method of assay for this bluish fluorescent pigment. It was suggested that the fluorescent compound was the methochloride of nicotinamide (322). However, it was shown by Ellinger and Coulson (323, 324) that synthetic nicotinamide methochloride was not fluorescent and only became so on treatment with alkali and solution in isobutanol. These authors isolated the nonfluorescent precursor of F_2 and showed it to be nicotinamide methochloride and suggested that F_2 was a mixture of two substances (1) F_2b, 6-hydroxy-1, 6-dihydro-1-methylpyridine-3-carboxylic acid amide, and (2) F_2a, is probably the α-carbinol which can be separated by adsorption on alumina (324) see also (325).

Ellinger (326) has recently shown that, on treatment of the precursor of F_2, nicotinamide methochloride, with alkali, a weak whitish-blue fluorescence is produced. Extraction of this pigment with isobutanol increases the fluorescence by as much as 400 times in intensity. This highly fluorescent compound is supposed to be the isobutanol ether of the carbinol.

Opinion is at present divided as to the significance of F_2 excretion for the evaluation of niacin metabolisim (327).

A. THE ASSAY OF N′-METHYLNICOTINAMIDE

The fluorimetric assay of N′-methylnicotinamide in urine has been worked out by Huff, Perlzweig and Tilden (328). These authors make use of the fact that N′-methylnicotinamide combines with acetone at an alkaline pH to yield a green fluorescent substance which is changed by acid to a more stable blue fluorescent compound.

The method is as follows:

Procedure. To 1.00 ml. of urine diluted to contain 0.3-1.5 γ of N′-methylnicotinamide, in fluorometer tubes graduated at 10 ml., are added 0.5 ml. of acetone (redistilled over $KMnO_4$) and 0.20 ml. of 6 *N* NaOH with mixing. After standing at room temp. for 5 min. 0.30 ml. of 6 *N* HCl are added, the mixture is heated in a boiling water bath for 2 min., cooled, 1 ml. of 20% solution of KH_2PO_4 is added, and the contents are diluted to 10.00 ml. A blank solution is prepared as above, simultaneously, but substituting 0.5 ml. of water for the acetone. For the standard, a recovery tube is prepared from the same urine diluted in such a way that 1 ml. contains 0.4-0.6 γ of added N′-methylnicotinamide and treated as above. The fluorescence, which is stable for days, is read in any fluorimeter equipped with the filters used for the determination of thiochrome. Highly pigmented urines low in N′-methylnicotinamide, and those giving high blank values, may be first decolorized with charcoal in a 2% acetic acid solution.

B. THE ASSAY OF F_2

The method developed by Najjar (329) for the estimation of the metabolic product of nicotinic acid F_2 is a modified procedure based on the method first described by Najjar and Wood (320).

The method consists of the following steps: The urinary pigments and extraneous fluorescent substances are removed by adsorption on charcoal, the F_2 is absorbed on permutit and eluted with KCl solution, the eluate is made alkaline and extracted with butyl alcohol.

The method is quoted in detail below:

A 25 cc. sample of fresh urine[1] is acidified with 1 cc. glacial acetic acid and 0.1-0.2 g. adsorbent charcoal[2] is added. The sample is stirred for a few seconds until the charcoal is thoroughly mixed and is then filtered through a rapid filter paper. Then 20 cc. of the clear or slightly yellowish filtrate are passed through a glass adsorption column[3] containing 3-4 g. activated permutit.[4] The rate of flow through the column should not exceed 2 cc./min. The column is then washed with 40-60 cc. distilled water with suction. Care should be taken to give the tip of the column a careful rinsing with distilled water in order to remove any possible contaminating fluorescent substances derived from the rubber suction tube. The column is then eluted with 25% KCl. The rate of flow of the KCl solution from the column should not exceed 1 cc./min.; 11 cc. of the eluate are collected, of which a 5 cc. aliquot is transferred into a glass-stoppered tube or separatory funnel; 13 cc. of distilled *n*-butanol are then added, followed by 1 cc. 15% NaOH, after which the container is immediately shaken vigorously for 2 min. This step is critical, since the necessarily strong alkali will destroy appreciable amounts of F_2 (methylnicotinamide derivatives) if allowed to stand before shaking. Another 5 cc. aliquot of the eluate is treated similarly except that no alkali is added. This serves as a blank.

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[1] Urine may be preserved for analysis by the addition of 2-3% of its volume of glacial acetic acid. The urine sample should contain no more than the equivalent of 50 γ N′-methylnicotinamide chloride. With higher concentrations, such as may occur after a load test, a smaller sample diluted to 25 cc. with 2% acetic acid should be used.

[2] The adsorbent charcoal supplied by Mallinckrodt Chemical Works, St. Louis, is excellent for this purpose. Satisfactory results are also obtained with Nuchar "T" and "W" supplied by West Virginia Pulp and Paper Co., Tyrone, Pennsylvania.

[3] Suitable adsorption columns can be purchased from E. Machlett and Sons, 220 E. 23rd St., N. Y. C. or can be made from standard glass tubing 8-10 mm. in diameter, drawn to a tip at the base. A small packing of glass wool is introduced at the tip to keep the permutit in place.

[4] Activation of permutit is done in the following manner: 500 g. of commercial permutit, 60-80 mesh (The Permutit Co., N. Y. C.) are boiled gently for 1 hr. in a solution of 25% KCl containing 5% acetic acid. After draining off the fluid, the process is repeated with a new lot of KCl and acetic acid. The permutit is then thoroughly washed with hot distilled water until it is almost chloride-free. It is then left to dry in an oven at about 100° C. Such permutit should not be used for more than a single determination. It may, however, be reactivated by boiling with KCl as above, after which it is tested for fluorescent substances by the usual elution procedure.

The samples are then centrifuged at low speed to separate the alcohol from the aqueous layer. The latter is drained off through a separatory funnel or pipetted off, and the butanol is cleared with a pinch of anhydrous sodium sulfate. The alcohol extracts are then allowed to stand for 20 min. to develop the full fluorescence of the carbinol-ether compound, after which fluorescence is measured by means of a fluorophotometer[5] or visually in a fluoricomparator.

When determinations are made with a fluoricomparator, the butyl alcohol extracts may be matched against standards prepared from known concentrations of N′-methylnicotinamide chloride.[6] The comparisons should, of course, be made in similar tubes containing similar quantities of fluid. We have found that standards treated with alkali and extracted with butanol to convert the N′-methylnicotinamide chloride into the fluorescent carbinol-ether are stable when kept on ice for over 10 days.

When using a fluoriphotometer, the instrument must first be standardized. Simultaneous readings are then made of the blank and of the alkali-treated sample. If A is the fluorescence (in galvanometer units) of the alkali-treated sample, B the fluorescence of the blank from the urinary eluate untreated with alkali, M the fluorescence of the butanol extract of a solution containing 10 γ methylnicotinamide chloride in 5 cc. 25% KCl, treated with alkali and butanol exactly as is done with the urine eluate, and B the fluorescence of a butanol extract of pure KCl, then:

$$\frac{A-B}{M-B} \times \frac{11}{5} \times \frac{26}{20} \text{ or}$$

$\frac{A-B}{M-B} \times 28.6$ = methylnicotinamide chloride in the original 25 cc. urine sample.

Standardization of the Fluoriphotometer. Before making fluorescence measurements the instrument should be turned on for 15 min. with all filters in place to allow the source of light to reach a stable maximum. It is not necessary to use N′-methylnicotinamide chloride for standardization each time the instrument is used, and it is usually more convenient to employ a quinine solution for that purpose. Once the quinine equivalent has been determined for a standard solution of N′-methynicotinamide chloride with a particular optical system, the quinine can be substituted for the daily standardization. In practice we employ a solution containing 20 γ quinine sulfate in 0.1 *N* H_2SO_4 for the routine setting of the instrument. For original standardization the methylnicotinamide butanol extract is first measured in the cuvette and the diaphragm or the amplifier of the instrument adjusted until it registers some arbitrary round number in galvanometer units. The quinine standard is then substituted and the reading in galvanometer units recorded. On subsequent occasions when the quinine standard is used, adjustment of the diaphragm or amplifier to reproduce this same galvanometer reading will reproduce the optical conditions obtained with the N′-methylnicotinamide chloride standard.

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[5] We have found the Pfaltz and Bauer instrument satisfactory, using filters UG2 for the exciting light and BG14 and GG3 for the fluorescent light. We have also used the Coleman instrument equipped with a B-1 filter for the exciting light and PC-1 filter for the fluorescent light.

[6] N′-methylnicotinamide chloride may be purchased from W. A. Taylor and Co., 7300 York Road, Baltimore, or may be prepared by the method of Karrer *et al.* (331).

The following is the method of assay described by Coulson, Ellinger and Holden (330).

The principle of the method is based on the separation of nicotinamide methochloride from urine by adsorption and elution, transformation of the substance into fluorescent derivatives (F_2) by treatment with alkali and extraction with isobutanol, and estimation of the concentration of F_2 in isobutanol by visual fluorimetry.

The assay is carried out as follows. The lower ends of thistle funnels of 4-5 mm. internal diameter are plugged with glass wool, and 1 g. Decalso (60 mesh) (Permutit Co., Ltd.) is washed into each tube. Five ml. or less of the urine diluted to 10 ml. are filtered through the column, which is then washed with 50 ml. distilled water and eluted with 14 ml. 25% (w/v) KCl solution. The eluate is collected in a dry tube and can be stored in the cold without deterioration. The eluate is divided into two equal portions which are transferred into glass-stoppered tubes of about 12 ml. capacity. Non-fluorescent isobutanol (2 ml.) is added to each, and 1 ml. 15% (w/v) NaOH to one to develop fluorescence. The tubes are at once shaken vigorously for 5 min., and, after separation of the layers, the isobutanol is pipetted off into identical non-fluorescent test-tubes, each containing about 100 mg. anhydrous Na_2SO_4.

The isobutanol extract of the neutral half of the KCl eluate is used as a reagent blank. The fluorescence intensity of the extract is compared with that of standards. The extracts are stable at room temperature in the dark.

Standards are prepared by passing a series of 10 ml. portions containing 1-12 γ/ml. of crystalline nicotinamide methochloride, prepared as described by Karrer *et al.* (331), in distilled water, through 1 g. Decalso columns. The columns are washed and eluted with 14 ml. 25% KCl solution in the manner described above. Seven ml. of each of the eluates are made alkaline and extracted with 2 ml. isobutanol. The separated extracts, dried with Na_2SO_4, are used as the standards. The tubes should be identical with those used for the extracts in the assay and the standards should be kept in the dark.

The isobutanol extracts are read in the filtered 366 mμ line beam of a high-pressure mercury lamp by naked-eye comparison of the unknown with the standards, in a dark room. With such standards, no instrument for measuring fluorescence intensity is necessary, apart from the high-pressure mercury lamp. To increase the accuracy of the assay, the results of three estimations with different amounts of urine should be averaged.

For large scale routine work, wooden racks supporting 12 thistle funnels were used. Sixteen tubes were shaken simultaneously in a box constructed for the purpose.

The following is a rapid method developed by Huff and Perlzweig (332).

The urines in such tests are best diluted 10-fold with 25% KCl solution. To a 10 ml. aliquot of the diluted urine in a test-tube, made alkaline with 2 or 3 drops of 10 *N* NaOH solution, 0.5 g. of Lloyd's reagent is added, the contents mixed for 1 min. by inversion, centrifuged for 2-3 min., and the supernatant fluid decanted. This procedure was shown to destroy completely the fluorescence of N′-methylnicotinamide, without affecting the other pigments of urine which are not adsorbed at the alkaline pH. Hence this proved to give the best blank values we have been able to obtain so far. An aliquot of 1-5 ml. of the diluted urine containing 3-15 γ and simultaneously an equal volume of the blank prepared as above are placed in 125 ml. separatory funnels, extracted with *n*-butanol. The butanol extracts are dried over anhydrous Na_2SO_4 and the fluorescence measured (333).

Mickelsen and Erickson (334) have examined the two assay methods of Najjar and his colleagues (329, 319) and that of Huff and Perlzweig (332) in connection with the excretion of F_2 in relation to dietary intake. They obtained radically different results by each of these methods, and it would appear that some of the ambiguities of the relationship between niacin intake and F_2 excretion may be ironed out by more refined methods of assay.

IV. Miscellaneous Physical Methods

1. Polarographic Methods

Polarographic methods of assaying the vitamins have not been worked out in any great detail with one exception, *viz.,* ascorbic acid.

In the case of the other vitamins the little work that has been carried out on the application of the polarographic method has been of a preliminary nature and applied to pure preparation of the vitamins. Like other physical methods the polarographic method suffers from the disadvantage that interfering substances are almost always present in crude concentrates of the vitamins.

The polarographic estimation of ascorbic acid is based on its oxidation at the dropping mercury electrode. In this it differs from other methods since no specific compound is used as an oxidant. Since the method is based on current voltage curves it is applicable to the determination of ascorbic acid, which possesses such well marked reducing properties.

Kodicek and Wenig (335) showed that dehydroascorbic acid was not reducible at the dropping mercury cathode, but a characteristic wave could be obtained at the dropping mercury anode. These authors applied this method successfully to fruit juices, using a phosphate buffer (0.066 *N*) of pH 7.0. In the case of ascorbic acid in tissues and in plant extracts the method failed because of interfering substances.

Schwarz (336; cf. 337) has used acetate-acetic acid buffer between pH 3.0 and pH 7.0. At pH 7.0, however, appreciable decomposition of the ascorbic acid was observed. This author used a buffer consisting of equal parts of 1 *N* acetic acid and 1 *N* sodium acetate, for the determination of vitamin C in fruit juices.

A polarographic method has recently been reported by Gillam (338) which is applicable to extracts of plants as well as expressed fruit juices. This author critically surveyed the buffer solutions which might be used in the determination and found that potassium biphthalate-sodium hydroxide buffer (pH 8.0) most nearly satisfied the requirements of pH, residual current curve, the stability of ascorbic acid in the supporting electrolyte, and buffer capacity.

The half-wave potential of ascorbic acid shifts towards more positive values with increasing hydrogen ion concentration and the pH of the supporting electrolyte must, therefore, be kept within a certain range.

Gillam used two supporting electrolytes in his determination. (a) Electrolyte I equal volumes of the phthalate buffer and 0.25% oxalic acid which gave a resulting pH of 4.6 and (b) Electrolyte II equal volumes of the phosphate buffer and 1.5% metaphosphoric acid which gave a resulting pH of 3.4.

Gillam's method follows:

Reagents. Potassium biphthalate buffer (pH 6.2), 500 ml. of 0.1 *M* potassium acid phthalate plus 470 ml. of 0.1 *N* NaOH, made up to 1 l.

Phosphate buffer (pH 8.0), 50 ml. of 0.066 *M* potassium dihydrogen phosphate plus 950 ml. of 0.066 *M* disodium hydrogen phosphate.

Oxalic acid solution, 0.25%; metaphosphoric acid solution, 1.5%.

Apparatus. The current-voltage curves were obtained by means of a Leeds and Northrup Electro-Chemograph. All the polarograms were obtained by using an external saturated calomel electrode as cathode, connected to the electrolysis cell through an agar bridge saturated with KNO_3.

All experiments were carried out in a water bath at 25.0° ± 0.2° C. The amount of mercury flowing from the dropping electrode per second, designated by m, was determined while dropping in air (339). The m and t values of the dropping capillary used were, respectively, 2.33 mg. of mercury/sec., and 3.0 sec. when the capillary tip dipped into the supporting electrolyte and no potential was applied to the mercury drop. The drop time, of course, varies with the nature of the supporting electrolyte and the potential of the dropping electrode, but it was found much more expedient, at the start of an analysis, to adjust the pressure on the mercury, so that $t = 3.0$ sec. in all the supporting electrolytes used. In all cases the drop time was also measured at the approximate potential at which the diffusion current was measured. Thus for supporting electrolyte I (biphthalate-oxalic) $t = 2.85$ seconds, for electrolyte II (phosphate-1.5% HPO_3) $t = 2.67$ seconds, respectively, when a potential of 0.19 and 0.26 was applied to the mercury drop. The value of $m^{2/3} t^{1/6}$ for the capillary used was 2.08.

Procedure. A given weight of the material to be analyzed is whipped in a Waring Blendor with a known volume of metaphosphoric or oxalic acid for 2-5 min. (340). A 2 or 5 ml. aliquot of the filtrate is withdrawn, an equal volume of the buffer solution is added, and the well-mixed solution is polarographed between -0.1 and $+0.3$ volt.

The diffusion current is then measured on the polarogram, corrected for the residual current of the supporting electrolyte, and the corresponding concentration of vitamin C is read off the calibration graph (see below).

If the concentration of ascorbic acid in the aliquot used is too great to give a satisfactory limiting current, the sample may be diluted with the supporting electrolyte being used or a lower sensitivity of the instrument may be employed. In analyzing solutions one merely adds a

1-5 ml. aliquot to an equal volume of the supporting electrolyte, and polarographs the solution. (Fig. 15.)

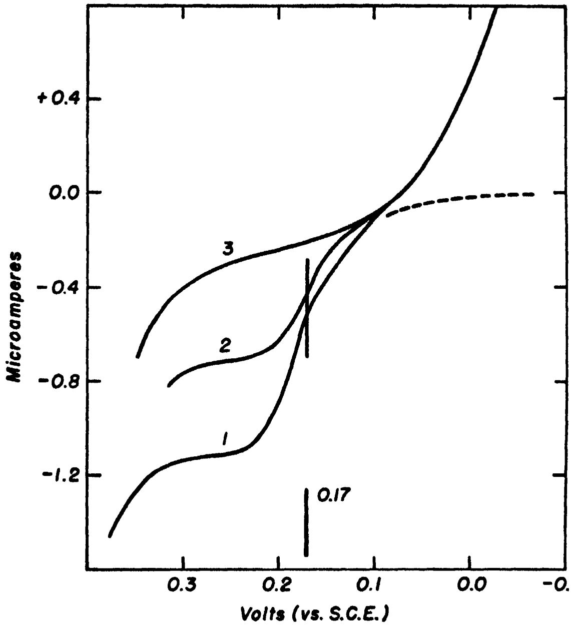

Fig. 15. Current-voltage curves of ascorbic acid in supporting electrolyte II at 25° C. (after Gillam (338)).

The instrument was calibrated by polarographing solutions containing known amounts of crystalline ascorbic acid, shown to have a purity of 98% by an iodine titration (Fig. 16). The residual current was determined for each supporting electrolyte by polarographing the solution between −0.1 and ± 0.3 volt at each range of the recorder, *e.g.*, 40, 28, 10, 5, 4, 3, and 2 microamperes. This corresponds to a range of 1/20 to full sensitivity of the galvanometer. The corrected diffusion currents were then obtained by measuring the distance between the limiting current and the residual current.

Oxygen was not removed from the solutions analyzed because it did not interfere with the ascorbic acid determinations and by eliminating the aeration procedure, considerable time was saved.

The value of the half wave potential was found by Gillam to be independent of the concentration of the ascorbic acid in the supporting electrolytes examined.

The following tables (Tables XX and XVI) taken from Gillam's article shows the reproducibility of his results by this method. The results obtained by this polarographic method were in good agreement with those obtained by the indophenol and photometric methods. Some modifications of the Gillam method have been suggested by Page and Waller (341).

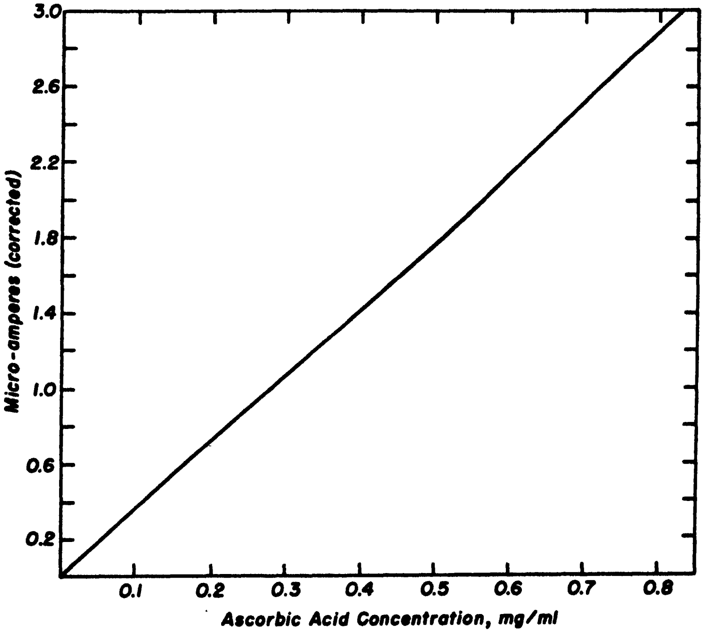

Fig. 16. Calibration curve for ascorbic acid in supporting electrolyte (after Gillam (338)).

A polarographic method for the determination of ascorbic acid in milk has been described by Perrin and Perrin (342).

The polarographic method has several advantages over the other methods of ascorbic acid determination, not the least of which is, that once the initial calibration is made, no further standardization of the solution is necessary. Extracts made with oxalic acid may be stored over considerable periods without loss of vitamin. Pigments do not interfere with the measurement of the half-wave potential.

Lingane and Davis, (343) have suggested that it would be possible to determine riboflavin by polarographic methods (344).

The diffusion current of riboflavin was shown by these authors to be directly proportional to its concentration in phosphate buffer at pH 7.2, over the range from 2 to 50 p.p.m.

TABLE XX

Reproducibility of Results

(γ ascorbic acid/ml. of solution)

Material		
Tomato		61.6
		60.1
		61.6
		60.8
	Mean	61.0
	Average deviation	0.6
	Coefficient of variation, %	1.0
Tomato		94.0
		94.0
		94.0
	Mean	94.0
	Average deviation	0.0
	Coefficient of variation, %	0.0
Orange		40.0
		40.0
		40.8
		39.6
	Mean	40.1
	Average deviation	0.4
	Coefficient of variation, %	1.0
Spinach		60.0
		60.0
		62.2
		58.0
	Mean	60.0
	Average deviation	1.0
	Coefficient of variation, %	1.6
Cauliflower		180.0
		182.0
		180.0
	Mean	180.6
	Average deviation	0.9
	Coefficient of variation, %	0.5
Pepper		137.6
		132.0
		130.0
		133.2
	Mean	133.2
	Average deviation	2.2
	Coefficient of variation, %	1.6

TABLE XXI

Pure Solution of Ascorbic Acid

Contained	Found
300	300
300	300
321	325
38	38
30	30
30	30
30	30
22	21.8
11.7	11.7

However, these authors only carried out assays on relatively pure samples of riboflavin. It has not as yet been determined whether this method can be applied to the assay of riboflavin in natural products.

A. VITAMIN K_1

Hershberg, Wolfe and Fieser (345) have studied the polarographic determination of vitamin K_1. It can be determined in aqueous isopropanol solution containing KCl. The typical curve obtained from K_1 shows a sharply defined wave at -0.58V. The wave was less well defined when LiCl was used in place of KCl.

B. MISCELLANEOUS

For data on other vitamins see (343). For a polarographic method for the determination of vitamin A see (346).

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Chemical Methods of Vitamin Assay

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No serious attempt was made to eliminate the slight differences in the presentation of the material. The resulting lack of complete uniformity was not considered as a major possible source of criticism. The sections dealing with vitamin A, carotene, thiamine, niacin, pyridoxine, and ascorbic acid were written by S.H.R., whereas P.G. was responsible for the rest—*Editor's Note.*

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I. Vitamin A and Carotene

1. Historical Development

The problems of vitamin A methodology are complicated by the circumstance that vitamin A is only one of several natural substances with the specific physiological effects of this vitamin. At least 9 different naturally-occurring provitamins A and 2 vitamins A are known and the work of isolating and identifying new forms continues. A detailed discussion of the provitamins A appears in the chapter on physical methods of vitamin assay. However, the relative biological potencies and distribution of these factors in nature are such that it often suffices to determine only β-carotene and/or vitamin A. Preformed vitamin A is not found in plant materials, but does occur in such animal products as butter, eggs, milk, and, above all, fish liver oils.

Quite recently, synthetic vitamin A has become a commercial reality, syntheses having been published by Van Dorp and Arens (1) and by Isler, Huber, Ronco and Kofler (2). The impact of synthetic vitamin A upon the analytical situations described below has not yet been thoroughly evaluated.

A. β-CAROTENE

The physicochemical determination of β-carotene is based upon the measurement, by colorimetric or spectrophotometric means, of the intensity of yellow color (absorption at 440 mμ) of this provitamin in a suitable extract. Three types of materials are commonly analyzed: 1) materials such as many pharmaceutical preparations which contain vitamin A as the only member of the "A" group; 2) materials which contain vitamin A and β-carotene as the chief sources of vitamin A activity; and 3) materials which contain appreciable amounts of various other carotenoids, with and without vitamin A activity, besides vitamin A and/or β-carotene. The choice of an analytical method is, therefore, determined by the type of material under investigation; no single standard method is necessarily applicable to or adequate for all products. Due to the very large number of publications concerning the determination of carotene during the last two decades, the following discussion will attempt no more than to highlight the principal trends in this field.

The major problem in carotene methodology has been the determination of β-carotene in the presence of other carotenoids. The first qualitative investigations of the occurrence of carotene in various materials relied largely on detection of the yellow color of this pigment (3), but the need for separation of carotene from other yellow pigments, notably the xanthophylls, soon became apparent. An early technique for such separation was proposed by Willstätter and Stoll (4), who employed the principle of biphasic separation. They showed that when the mixed carotenoids in petroleum ether are shaken with 90% methyl alcohol, the xanthophylls enter the alcohol phase while the provitamins A (hydrocarbons) remain behind. This method was widely used for some time, especially for grass and forage (*e.g.*, 5-7) and still finds application when β-carotene constitutes all or nearly all of the provitamin A fraction. Methyl alcohol is usually employed (8), although diacetone alcohol has also been recommended (9, 10). The second method, which is more generally applicable and more specific, was pioneered by Tzvyet (*cf.* 11) and consists of the fractionation of the carotenoids upon a suitable adsorption column. A number of adsorbents have been used, among them magnesium hydroxide (12), aluminum oxide (13-16), Hyflo Super-Cel (17), magnesium oxide (18-19), mixtures of magnesium oxide and carbonate (21) and of magnesium oxide and Hyflo Super-Cel (22, 23), calcium hydroxide (24, 25), sodium carbonate (26, 27), dibasic dicalcium phosphate (28), a mixture of this adsorbent with calcium carbonate

(29), starch (30), and various other mixtures (31 and 32). Van Eekelen *et al.* (33) found that the losses incurred in the chromatographic (Al_2O_3 or $Ca(OH)_2$) separation of carotene from other carotenoid pigments could be avoided by using as solvent a mixture of 10 parts ether and 15 parts benzene.

In most cases, the adsorbent must be suitably activated before use. A unique variation of this activation procedure has been proposed recently by Müller (34, 35), who used adsorption columns packed with three layers of aluminum oxide with graded activities in order to separate β-carotene from vitamin A alcohol and vitamin A esters and to separate vitamin A, vitamin D and β-carotene. The methods of choice for a variety of products, selected by the Association of Official Agricultural Chemists (36), are reproduced in detail in the chapter on physical methods of vitamin assay, which deals more extensively with other chromatographic procedures as well (p. 99, 104).

Besides the central problem of carotene methodology, which deals with the separation of carotene from other carotenoids, the question of the best method of extraction of the vitamin from natural materials has concerned various investigators. A recent trend in the extraction of carotene from plant tissues has been to blend the material directly with petroleum ether in a Waring blendor (37, 38). Saponification of the sample before extraction of carotene with petroleum ether is preferred by others (21), while, in the case of vegetable oils, simple solution in petroleum ether has been proposed (13). Shaking with a mixture of naphtha and alcohol has been found to be sufficient to extract carotene from ground whole meat (39).

Saponification is included in the analytical method when both β-carotene and vitamin A are to be measured in a sample. The usual colorimetric procedure runs as follows: saponification of the sample; extraction of the unsaponifiable fraction with petroleum ether; chromatography to eliminate xanthophylls and other noncarotene carotenoids; measurement of the yellow color of the eluate (attributed to β-carotene) at 440 mμ; evaporation of a portion of the eluate and solution of the residue in chloroform, addition of antimony trichloride reagent to form a blue color, read at 620 mμ, which includes both vitamin A and β-carotene, and correction of the total blue color for the amount of β-carotene present to obtain the concentration of vitamin A.

a. Correction of Total Blue Color for β-Carotene (cf. discussion of Carr-Price reaction in section on Vitamin A). When the early work on the determination of vitamin A was extended from fish oils to other materials, it became apparent that $SbCl_3$ produces colors with a wide range of sterols and carotenoids (40). Shortly thereafter, the colorimetric characteristics of the blue color formed with such carotenoids as β-carotene were established (41). The problem of determining preformed vitamin A in the presence of β-carotene has been given considerable attention by the workers in the field. In adapting the Carr-Price reaction to the Evelyn colorimeter, Dann and Evelyn (42) measured the yellow color of the final chloroform extract at 440 mμ and subtracted from the total blue color a percentage fraction which was obtained by correlating yellow and blue color intensities of standard β-carotene. This method was based on the premise that there is a constant quantitative relationship between the absorption of β-carotene at 440 mμ and the blue color formed with $SbCl_3$. As presented by these authors, and adopted by Koehn and Sherman (43), who used a correction graph rather than a percentage factor, the method represents only a first approximation, since the yellow color of various extracts is usually due to a mixture of carotenoids and not to β-carotene alone.

Johnson and Baumann (44) have recently studied the variations in the intensity and rate of formation of the blue color formed when $SbCl_3$ reacts with derivatives of carotene. They observed wide variations in the blue to yellow ratios obtained with carotene fractions from natural materials. Oxidation products of carotene had a much greater effect upon the $SbCl_3$ reaction, both qualitatively and quantitatively, than carotene itself. Therefore, if reliable assays for vitamin A are desired, these components must be separated by chromatography or by extraction with methanol. A valid correction may be calculated when preliminary chromatography of the extract yields a fraction free of noncarotene carotenoids (9, 24, 26). In some cases, preliminary chromatography has been omitted entirely (45) or replaced by methyl alcohol extraction as discussed above (46). Another change in procedure has been the measurement of the color of the petroleum ether extract rather than of the final chloroform solution, followed by evaporation of the ether and resolution of the residue in chloroform for colorimetry with $SbCl_3$. Fig. 1 shows a typical graph establishing the relationship between the absorption of β-carotene in petroleum ether at 440 mμ and the blue color formed with $SbCl_3$.

A simple procedure for the correction of total blue color for β-carotene is based upon the usage by Oser, Melnick and Pader (47) of the fact that the maximum color produced by vitamin A with $SbCl_3$ occurs after about 4 sec. and fades steadily and rapidly from that time on, while the maximum color produced by β-carotene with the same reagent remains stable for at least 2 hr. These authors showed that vitamin A and carotene, in solution together, react in an additive manner, and therefore proposed that the blue color remaining 2 hr. after the addition of $SbCl_3$

to such a mixture be subtracted from the total blue color formed within the first 4 sec. in order to obtain a measure of preformed vitamin A. Oser and coworkers pointed out that this procedure is subject to the same general criticism as the Dann and Evelyn method of correction, namely, that it is nonspecific. In this case, the lack of specificity affects the blue color reading after 2 hr. of fading, which is assumed to be due only to β-carotene but actually may be a measure of a mixture of carotenoids. Another variable in this procedure was identified by Caldwell and Parrish (48), who showed that the intensity of the incident light influences materially the rate of fading of the color. On the basis of these investigations, Brew and Scott (29) developed a standardized

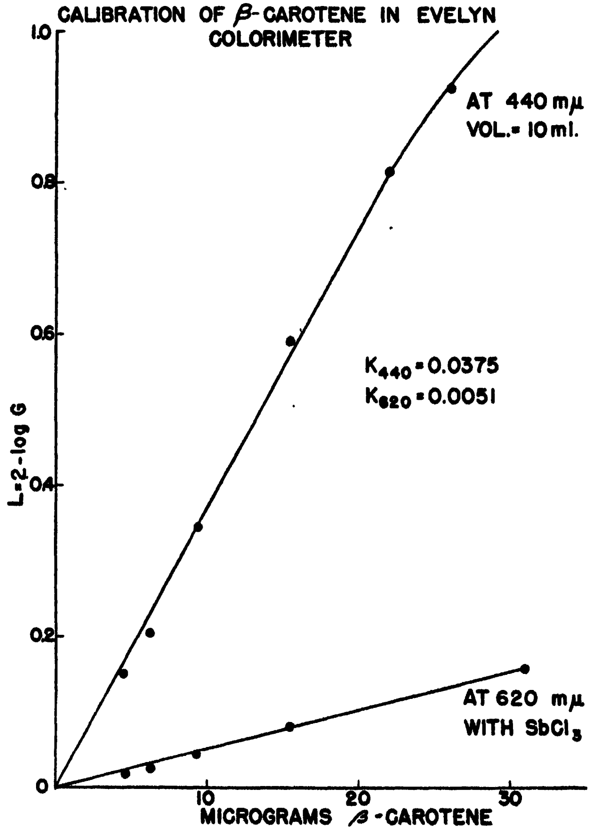

Fig. 1. Relationship between absorption of β-carotene at 440 mμ and the blue color formed with antimony trichloride.

method of analysis for vitamin A in feeds which employed the fading technique, but also included chromatography to keep the method more highly specific.

The effect of temperature on the development and fading of the $SbCl_3$ colors of vitamin A and common carotenoid pigments has been studied by Caldwell and Hughes (49). They observed that the maximum color with vitamin A develops almost instantaneously and is independent of the temperature. No major differences were found in the intensity or in the rate of fading of the colors with vitamin A alcohol, acetate, or natural esters. As the temperature rises, marked increases occur in the rate of fading of the vitamin A color. Normal variations in room temperature were deemed insufficient to affect the vitamin A determination seriously, provided that readings are taken within a few seconds after mixing the reactants. Fading curves of the carotenoids—α-carotene, β-carotene, γ-carotene and lycopene—are characterized by a color development period ranging from a few seconds in the case of β-carotene, to several minutes in the case of lycopene, followed by a decrease to a plateau value. Both the development and fading appear to be accelerated slightly by higher temperatures (35° and 50° C.). In the case of the carotenols, cryptoxanthin curves closely resemble those of the above carotenoids, whereas lutein and zeaxanthin are characterized by slow development of color, strongly influenced by temperature, and an abrupt drop to plateau values at higher temperatures. These considerations point up the difficulties involved in determining vitamin A in the presence of high concentrations of carotenoids. Since the rate of color development in the carotenoid-antimony trichloride reaction is suppressed by low temperatures, these authors suggest the possibility of using special low temperature techniques for satisfactory determination of vitamin A in the presence of large amounts of the common carotenoid pigments.

Chromatography can also be applied in another way. Instead of separating vitamin A and β-carotene from all other carotenoids, and then correcting the total blue color for β-carotene to obtain a measure of vitamin A, a chromatographic adsorbent can be chosen which separates β-carotene from vitamin A and permits separate determination of each fraction. This approach was first proposed by Karrer and Schoepp (50) who used alumina. More recent investigations indicated the successful use of charcoal (51), bone meal (52, 53), layers of aluminum oxide with graded degrees of activation (34, 35), a mixture of calcium hydroxide and "Super-Cel" (24) and dibasic calcium phosphate (29). A more extensive discussion of this technique can be found in the chapter on physical methods of vitamin assay. Boyer *et al.* (54) proposed that the separation of carotene from vitamin A be carried out by making use of the differential solubility of these compounds in 50-60% ethyl alcohol. This method has not been explored further. From a developmental point of view, the direct chromatographic separation appears to be the most advanced; the Dann and Evelyn correction with preliminary chromatography, however, still enjoys widespread usage.

B. VITAMIN A

The color reactions which have been proposed during the last 25 years for the determination of vitamin A are based largely on 3 types of reactions: reactions with phenols, with acids, and with inorganic chlorides which have an acidic reaction in aqueous solution (1, p. 78).

1) Reactions with phenols alone may be illustrated by the work of Fearon (55) with pyrogallol. The phenolic principle has also been used in combination with the other two types of reactions (*v.i.*).

2) Reactions with various acids have been put forward from time to time. Ueno (56), Drummond and Watson (57), and Kobayashi and Yamamoto (58) proposed concentrated H_2SO_4 as a reagent; the latter investigators also worked with H_3PO_4. Fearon (55) suggested trichloroacetic acid. A number of other acids, among them molybdenum phosphotungstic acid (59), and chloric acid in the presence of phenol and guaiacol (60), have been investigated. Recently, interest has again been aroused in the reaction with H_2SO_4 by qualitative experiments with superfiltrol and other acid earths (61-65).

3) The reaction with inorganic chlorides in chloroform has been applied to the following: $AsCl_3$ (66), $AlCl_3$, $FeCl_3$, $SnCl_4$, and $SbCl_3$ (67). Another reaction based on a similar reagent has recently been proposed independently by Sobel and Werbin (68) and by Feinstein (69), and its comparative merits and limitations have been investigated by Sobel and Werbin (70-73). The reagent, activated glycerol 1, 3-dichlorohydrin, is more stable and less susceptible to interference by moisture, and forms a color which fades less rapidly than that produced with $SbCl_3$. On the other hand, the reaction is less sensitive and requires correction for carotene in the same manner as the $SbCl_3$ reaction.

The reaction of vitamin A with $SbCl_3$ in chloroform has been the most widely-used colorimetric method for the vitamin A group ever since it was established by Carr and Price (67). The literature on the Carr-Price reaction is very extensive; it is proposed here briefly to trace its developmental history. The early investigations were conducted with various types of vitamin A concentrates in the form of natural or concentrated fish liver oils. The reaction was usually carried out between the reagent and a direct dilution of the fish oil in chloroform. It soon became apparent, however, that the reaction carried out in this way was irregular and that the relationship between color intensity and concentration was nonlinear. The fish oils used as sources of vitamin A were shown to contain various inhibitors of the $SbCl_3$ reaction (74-76). Saponification of the oil, followed by ether extraction of the nonsaponifiable fraction prior to colorimetry was recommended (74, 77, 78), and soon adopted universally. Some inhibitors, principally unsaturated fatty acids (79, 80), were found to accompany the vitamin into the nonsaponifiable fraction, but the majority of inhibiting substances were eliminated by this treatment. The developments leading to the adoption of saponification and the experimental conditions needed for satisfactory execution of saponification, extraction, and colorimetry have recently been reviewed and studied critically by Benham (81).

The chief problem presented by the Carr-Price reaction is the rapid fading of the blue color. Before the development of photoelectric colorimeters, the measurement of the color intensity by visual means was difficult because of this instability of the color. Various attempts were made to stabilize the color by the addition of such substances as catechol (82), guaiacol (83), and acetic anhydride (84). Notevarp and Weedon (85) have studied and summarized many of these early developments, including extensive spectrophotometric data which showed that the true maximum of the absorption curve of the color complex lies at 618 mμ.

A satisfactory practical solution of the problem of measuring the fleeting color was achieved by Dann and Evelyn (42), who adapted the measurement to the Evelyn photoelectric colorimeter (86). By virtue of the short galvanometric lag of the instrument and sufficiently specific filters, they secured greater precision, sensitivity, and objectivity in measuring the color. Armed with this technique, Dann and Evelyn reinvestigated the quantitative aspects of the reaction. Using the *micro* unit of the colorimeter, they found that the maximal color intensity occurs at 10 sec. with fresh $SbCl_3$ reagent, diminishes as the reagent ages. The experience in many laboratories has been that the maximum in the *macro* unit of this colorimeter occurs at 4 or 5 sec. with fresh reagent, slightly sooner with aged reagent. The time difference probably arises from a variety of factors, including the relative intensities of incident light (*cf.* Caldwell and Parrish (48)). Other factors were studied critically by Dann and Evelyn: saponification of all but very high potency samples was strongly recommended; the application of Beer's law to the blue solution was studied, and the precision of the method established; various experimental conditions, such as the concentration and purity of the reagent, time of reading and temperature, were fixed. An ingenious "dynamic" method, which permits the measurements to be made on a flowing mixture, so that the time of observation relative to the time of mixing is controlled and observations are unaffected by the transient nature of the reaction, has recently been described (87).

The choice of an adequate standard substance and the conversion of chemical measurements into units of biological activity, have been matters of great difficulty and uncertainty in the case of vitamin A. A discussion of standards, conversion factors, biological considerations and unitage falls outside the limits of this chapter. Excellent studies and summaries of these problems are available (88-91), in addition to the discussion in the chapter on physical methods of vitamin assay.

Following the work of Dann and Evelyn, the efforts of many investigators were devoted largely to working out special problems encountered in the application of the photoelectric method to various types of samples. Oser, Melnick and Pader (92) applied the $SbCl_3$ method to the analysis of fish liver oils and found good agreement with spectrophotometric values on fresh samples but increasingly large discrepancies on samples exposed to oxidation. The use of methylene chloride rather than ethyl ether for the extraction of vitamin A in soupfin shark liver oils was advocated by Tompkins and Bolomey (93). A rapid method for the analysis of vitamin A in liver has recently been proposed by Gallup and Hoefer (94). Zscheile and coworkers (46) proposed a colorimetric method for the assay of butterfat, which included biphasic separation with methyl or diacetone alcohol to remove xanthophylls. A rapid extraction procedure was described by Boyer *et al.* (45) for the determination of vitamin A and carotene in milk. Bessey *et al.* (95) have developed a method for determining vitamin A and carotene in small quantities of blood serum.* Oser (96) reported a method for the determination of vitamin A in fortified margarine which required the availability to the analyst of an unfortified "blank" sample of margarine for suitable corrections. The destructive irradiation technique as used in many cases where no "blank" sample is available, is discussed elsewhere. The recent investigation of Bowen and coworkers (97) points up difficulties encountered in the analysis of margarine. Chromatographic procedures have been described by Berl and Peterson (9) for butter, by Mann (52) for egg yolk, by Wilkie and DeWitt (32) for oleomargarine, by Cooley and coworkers (26) for mixed feeds, by Goodwin and Morton (53) for butter and margarine, by Brew and Scott (29) for mixed feeds and feedstuffs, and by Thompson *et al.* (24) for dried whole eggs. Glover, Goodwin and Morton (98) have described a chromatographic method for separating free and esterified vitamin A.

Oser, Melnick and Pader (47) proposed several modifications which help to give more reasonable results in routine determinations on a wide variety of foodstuffs. These modifications include: 1) the use of an internal standard to correct for effects on the blue color due to inhibitors, reagent variations and temperature variations; 2) a supplementary reading at 720 mμ to correct for turbidity, which may occur in the formation of the blue color; 3) a supplementary reading at 620 mμ without the addition of $SbCl_3$, to correct for extraneous color which may be present in the chloroform solution of the unsaponifiable.

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* p. 297.

Some light has recently been thrown on the extent of interference in the Carr-Price reaction by degradation products of vitamin A, which are biologically inactive. Embree and coworkers (99, 100) identified 4 dehydration or oxidation products of vitamin A which give a considerable amount of blue color with $SbCl_3$. Expressed in terms of $E^{1\%}_{1\,\text{cm.}}$ calculated on a molar basis, the relative intensities of the blue color formed were reported as follows.

Crystalline vitamin A	4800
Anhydro vitamin A	5500
Isoanhydro vitamin A	3200
Subvitamin A	310
Anhydro subvitamin A	300

That the progressive formation of these degradation products during aging tends to make Carr-Price values too high has been stated by Oser *et al.* (92) and others (101). On the other hand, Oser *et al.* (91) have recently evaluated comparative values by the colorimetric, spectrophotometric, and biological methods of assay of fish liver oils (*cf.* Table I) and other products, and come to the conclusion that the colorimetric method is generally more specific, simpler, and more reliable for many types of samples.

Sobel and Werbin (73) have made an extensive comparison of vitamin A determinations in fish liver oils by the glycerol dichlorohydrin, Carr-Price, and ultraviolet absorption methods. Good agreement was observed between the first two methods, whereas the ultraviolet absorption method gave appreciably higher values for both the whole oils and the unsaponifiable fractions.

2. METHODS OF ANALYSIS

The following methods are presented in detail.

1). ***Vitamin A in high potency or enriched materials.*** **This is essentially the method of Dann and Evelyn (42) with modifications proposed by Oser, Melnick and Pader (47).**

2). ***Vitamin A in mixed feeds and feedstuffs.*** **This method was devel-**

TABLE I

Per Cent Deviation of Nonbiological Estimates of Vitamin A Content from Bioassay Values[a]

		Whole Oil		Unsaponifiable Fraction	
Sample	Bioassay Value	Spectro-photo-metric[b]	Colori-metric[c]	Spectro-photo-metric[b]	Colori-metric[c]
	U.S.P. units/g	*per cent*	*per cent*	*per cent*	*per cent*
5	225,400	— 4.3	— 4.3	— 5.7	— 5.9
19	68,860	— 5.7	— 1.7	— 5.4	— 13.5
23	207,800	— 6.6	— 17.2	— 7.2	— 21.1
26	144,000	30.0	26.4	33.3	15.2
31	960,000	26.2	26.0	20.8	1.9
36	104,000	39.7	31.5	34.2	16.1
37	270,000	32.6	33.0	26.7	14.1
40	158,800	19.8	15.6	16.9	7.7
47	1,170,000	12.5	13.0	5.5	— 0.9
64	197,000	— 2.1	— 7.2	— 6.1	— 13.9
83	301,400	31.7	27.7	24.1	18.1
93	335,000	17.7	4.4	17.3	1.1
Av.		15.96	12.27	12.87	1.58
Standard deviation		16.88	17.10	15.91	13.17

[a] From Oser *et al.* (91).

[b] $E_{328} \times 2000$.

[c] Standard = distilled esters (Sample 5) ($E_{328} \times 2000 = 215{,}600$).

oped by Brew and Scott (29) and is notable for its system of correction for nonvitamin A colors.

3). *Vitamin A by the glycerol dichlorohydrin method.* This colorimetric procedure was devised by Sobel and Werbin (71, 72) and illustrates the use of a reagent other than $SbCl_3$.

4). *Vitamin A and carotene in whole dried eggs.* This method was established by Thompson *et al.* (24) and exemplifies a recent satisfactory solution of the problem of analyzing natural, low-potency foods containing appreciable amounts of cryptoxanthin.

5). *Carotene in alfalfa, sweet potatoes and carrots.* This is a recent chromatographic procedure which was elaborated by Kemmerer (102) and tested by collaborative studies; it relies entirely on chromatography for the separation of the carotenoids from the xanthophylls and from each other.

6). *Vitamin A and carotene in butter.* This method, developed by Berl and Peterson (9), makes use of phasic separation with diacetone alcohol for the removal of xanthophylls.

7). *Rapid method for carotene in vegetable oils.* This procedure was developed by Bickoff and Williams (13) and provides for chromatographic analysis without saponification.

8). *Rapid extraction procedure in the determination of Vitamin A and carotene in milk.* This method, developed by Boyer *et al.* (45), does not employ either phasic separation or chromatography, but may be applied to cow's milk since β-carotene constitutes most of the carotenoid fraction in this material.

A. DETERMINATION OF VITAMIN A IN HIGH POTENCY OR ENRICHED MATERIALS

After Dann and Evelyn (42) and Oser, Melnick and Pader (47).

a. Reagents.

Ethanol, 95%, U.S.P. or 2B, may be used without purification.

50% aqueous KOH, equal weights of KOH (C.P.) and water.

Petroleum ether, Reagent Grade "Benzin," boiling point range 30-75° C.

Sodium sulfate, C.P. Anhydrous, "Special for Nitrogen Determination."

Chloroform, Reagent Grade, stored in a dark bottle.

Antimony Chloride in Chloroform. 20-23% by weight, prepared by dissolving contents of a previously unopened 1 lb. bottle of antimony chloride (C.P.) in 1500 ml. of chloroform with gentle warming; after cooling, the solution is filtered through No. 12 Whatman filter paper and stored in brown glass-stoppered bottles in a dark cool place.

Vitamin A standard—The Reference Standard of the United States Pharmacopeia, adopted January 1, 1948, is a solution of crystalline Vitamin A acetate in cottonseed oil contained in a "low-actinic" gelatin capsule. This oil solution has a biological potency of 10,000 U.S.P. units of Vitamin A/g. Each capsule contains 250 mg. (± 1 mg.) of the oil solution, thus having a potency of 2500 units per capsule. The variation in weight of capsule contents is less than 0.5%, making it possible to use the complete content of a capsule without weighing.

The capsules are purchased from U.S.P. Reference Standards, E. Fullerton Cook, Revision Chairman, 4738 Kingsessing Avenue, Philadelphia 43, Pa.

b. Apparatus.

A direct-reading photoelectric colorimeter with interchangeable test tubes and 620 and 720 mμ filters. The direct-reading galvanometer must be critically damped, and have a short period. (The conditions of the assay are described for the Evelyn photoelectric colorimeter, or instruments of similar sensitivity.)

A micropipet, accurately calibrated to deliver 0.1 ml. between two marks.

A 9 ml. automatic pipet. Delivery should require not more than two seconds (*cf.* Fig. 2).

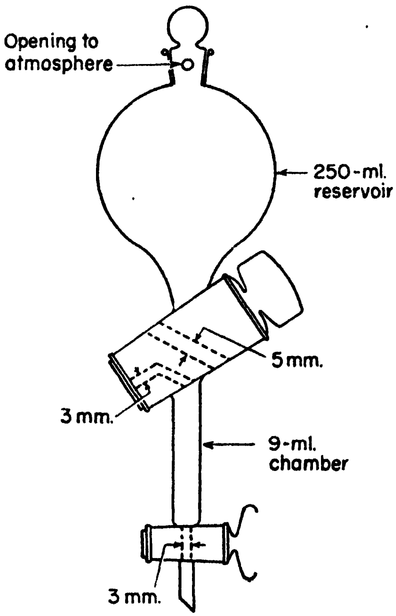


Fig. 2. Rapid-delivery pipette for Carr-Price reaction (after Oser *et al.* (47)).

c. Procedure.

A quantity of sample, containing at least 50 U.S.P. units of vitamin A, but preferably not larger than 10 g., is added to 75 ml. of ethanol in a 250 or 350 ml. amber refluxing flask; 10 ml. of aqueous 50% KOH are added, and the mixture is refluxed on a hot plate for 0.5 hr., or until mechanical disintegration has occurred. After cooling, the mixture is transferred to a 500 ml. separatory funnel with 3 × 20 ml. (total 60 ml.) of water. The solution in the separatory funnel should consist of 50 ± 5% ethanol.

The unsaponifiable material is extracted with 4 portions of petroleum ether (60, 50, 40, 30 ml., resp.), shaken for 2 min. each time, with intermittent release of pressure. After the first extraction, the alcoholic layer is run off into a second 500 ml. separatory funnel in which the remaining extractions are carried out.

The pooled petroleum ether extracts are washed 3 times with 50 ml. portions of water, the first portion being run down the sides of the separatory funnel without shaking. (This often helps avoid the formation of emulsions.) The washed extract is transferred to a 250 ml. amber Erlenmeyer flask with a spout. About 5 g. of anhydrous sodium sulfate are added slowly while the flask is swirled. The petroleum ether is dry when single crystals of sodium sulfate remain dispersed through the liquid during swirling. The dried petroleum ether extract is filtered through No. 41 Whatman filter paper into a second 250 ml. amber Erlenmeyer flask with a spout. Several small portions of petroleum ether are used to accomplish quantitative transfer. The extract is evaporated on a steam bath to a volume of about 5 ml. under a steam of nitrogen. The last few ml. are blown off by nitrogen at room temperature, leaving the residue cool and protected from oxidation. The residue is immediately taken up in chloroform and transferred to a suitable volumetric flask. The final chloroform solution, obtained by subdilution if necessary, should contain 10-25 U.S.P. units/ml.

Colorimetry. The center setting of the instrument (using the 620 mμ filter) is obtained by setting the galvanometer at 100 with a tube containing 1 ml. of chloroform and 9 ml. of the $SbCl_3$ reagent. Then to another tube containing 1 ml. of the chloroform extract of the unknown, 9 ml. of the reagent are added from the automatic pipet and the minimal constant galvanometer reading is recorded (usually within 5 sec. after the addition of the reagent). If the galvanometer reading is less than 20 or more than 70, the final chloroform extract should be taken sufficiently more or less dilute and colorimetry performed again, if maximal precision is desired.

If the chloroform extract of the unknown is colored, a blank correction is made by measuring the color intensity at 620 mμ of 1 ml. of the extract diluted to 10 ml. with chloroform.

To correct for turbidity, produced occasionally during the course of the reaction, the color is developed in a fresh aliquot, and the reading after 5 sec. noted, using the 720 mμ filter. The center setting for the 720 filter must first be determined as above.

Two methods are available for the conversion of the chemical units as read on the galvanometer to units of biological activity. In both methods, the first step is to convert all galvanometer readings (G) to photometric densities (L) according to

$$L = 2 - \log G.$$

The first method of calculation involves the determination of a *calibration constant* by means of a calibration curve. The following illustrates the steps.

0.4581 g. of the U.S.P. Vitamin A Reference Standard was weighed into a beaker and transferred to a 25 cc. volumetric flask with chloroform. Subdilutions were made from this solution.

0.4581 g. of Reference Standard = 4,581 U.S.P. units
Dilution 1 (to 25 ml.) = 183 units/ml.

The following table shows the various subdilutions of dilution 1 and the corresponding galvanometer readings.

Subdilutions	Vitamin A (units/ml.)	G	L	K[a]
4 → 25	29.2	21^2	0.668	0.0229
3 → 25	21.9	31^0	0.509	0.0232
5 → 50	18.3	36^2	0.438	0.0239
2 → 25	14.5	45^1	0.344	0.0237
3 → 50	11.0	54^0	0.268	0.0242
2 → 50	7.0	67^1	0.172	0.0246
				Av. = 0.0238

[a] Calibration constant, $K = \frac{L}{C}$

The photometric densities (L) were plotted against concentrations (c). The resulting calibration curve is practically a straight line between the concentration limits of approximately 10 and 25 U.S.P. units/ml. Two simultaneous determinations of the calibration constant in the same manner as above yielded the values 0.0238 and 0.0240, as an indication of the reproducibility of the procedure. In the calculation of the potency of any unknown, the photometric density, corrected for extract color and turbidity, is multiplied by the dilution factor and divided by the calibration constant, according to:

$$\frac{L_{\text{corr.}}}{K} \times D = \text{U.S.P. units/sample.}$$

This method of calculation is satisfactory for concentrates, pharmaceuticals, and other samples of fairly high potency.

For low potency foods, Oser, Melnick and Pader (47) have proposed an *increment procedure,* as follows. From the vitamin A standard described above, a chloroform solution containing 100 U.S.P. units of vitamin A/ml., is prepared as a working standard. To a tube containing a 1 ml. aliquot of the chloroform extract of the unknown (the L value of which has previously been determined as above), exactly 0.1 ml. of the standard solution is added from the micropipet, followed by 9 ml. of the $SbCl_3$ reagent, and the minimal constant galvanometer reading is noted. The equation used for calculation is:

$$\left(\frac{A - C - D}{1.01\ B - A}\right) \times 10 \times \text{dilution factor} = \text{U.S.P. units/sample where}$$

$A = L$ of the extract of the unknown
$B = L$ of extract $+$ 10 U.S.P. units of standard
$C = L$ of color blank at 620 mμ
$D = L$ of turbidity blank at 720 mμ.

This method of calculation proposes to correct for the presence of inhibitors of the color development, for temperature effects, and for variations in the reagent.

d. Notes on the Procedure.

1) It may be noted that a considerable excess of KOH for most samples is present in the saponification mixture. Benham (81) has discussed the variations in saponification procedure used by various investigators. A comparative table of saponification mixtures is given in the chapter on physical methods of vitamin assay. Since an excess of KOH, over and above that indicated by the saponification number of the fat, does not affect vitamin A, and since the difficulties commonly encountered in ethyl ether extractions due to excess KOH are avoided by the use of petroleum ether, a large excess of KOH, sufficient to saponify large amounts of fat, is not contraindicated.

2) The use of petroleum ether rather than ethyl ether for the extraction of the unsaponifiable fraction has been advocated by some investigators, rejected by others (*cf.* (81)). The satisfactory use of either solvent is indicated by the fact that one of the two methods presented here makes use of petroleum ether, the other of ethyl ether. Since the extraction problem is one of partition, the percentage of alcohol in the saponified mixture prior to extraction becomes of paramount importance. We have found petroleum ether extractions satisfactory when the saponificate contains 50 ± 5% ethanol. In the method which follows, the saponificate contains 22% methanol before ethyl ether extraction. The following advantages may be cited for the use of petroleum ether in a general routine procedure: the excess of KOH is not critical (see note 1); no emulsions at the interface are encountered in the extraction of a mixture containing 50 ± 5% ethanol and no complications arise during the washing and drying steps.

3) The use of amber glassware throughout this procedure has been recommended (47, 104) but need be adhered to rigorously only if direct sunlight cannot be avoided in the laboratory. Recently, Gallup and Hoefer (94) reported that exposure to bright daylight for 5 hr. caused considerable losses of vitamin A. If the extractions are carried out with

reasonable speed, however, the use of clear glass separatory funnels is safe for many types of samples.

B. DETERMINATION OF VITAMIN A IN MIXED FEEDS AND FEEDSTUFFS

After Brew and Scott (29)

a. Reagents.

Antimony trichloride reagent, 90 g. of reagent grade $SbCl_3$ in 240 ml. of reagent grade chloroform.

Aqueous KOH, 50 g. of reagent grade pellets in 50 ml. of distilled water.

Calcium phosphate, dibasic, should be tested to retain vitamin A and pass β-carotene (Mallinckrodt A. R. grade).

Skellysolve F, petroleum ether, boiling range 30-60° C.

Vitamin A standard solution, prepared from U.S.P. Vitamin A Capsules.

b. Apparatus.

A *direct reading photoelectric colorimeter* with interchangeable test tubes and 620 mμ filter. Brew and Scott (29) used the Evelyn photoelectric colorimeter.

c. Procedure.

Extraction. Place a weighed 10 g. sample of feed in a 20 × 80 mm. paper extraction thimble and extract for 4 hr. in a Butt-type extraction tube with 35 ml. of Skellysolve F placed in a 100 ml. extraction flask. Evaporate off the solvent from the extract under a vacuum, add 30 ml. of 95% ethyl alcohol and 3 ml. of aqueous KOH solution, and bring to boil in a water bath. Cool and wash contents of flask into a 250 ml. separatory funnel with a minimum amount of Skellysolve F. Add 30 ml. of distilled water and sufficient Skellysolve F to make 25 ml. total volume of the latter. Shake and draw off the lower phase after settling, and transfer the Skellysolve phase to a clean 125 ml. flask. Repeat the extraction of the aqueous phase with three more 25 ml. portions of Skellysolve and combine the extracts in a clean 250 ml. separatory funnel. Wash free from alkali by repeated washings or with a continuous spray washing device. Filter the alkali-free extract into a 300 ml. long-necked round-bottomed flask through about 15 g. of anhydrous sodium sulfate on a filter paper, and wash the filter paper with additional solvent.

Purification. If little or no color is present in the final extract, this step may be omitted. If an appreciable amount of colored pigments is present, evaporate the extract down to a 5 ml. volume under a vacuum and wash into a chromatographic column with a minimum volume of Skellysolve.

Prepare a 16 × 150 mm. chromatographic column with a 7.5 cm. (3 in.) layer of dibasic calcium phosphate, topped by a 2.5 cm. layer of calcium carbonate. The dibasic calcium phosphate should be tested prior to use and shown to retain vitamin A without destruction and to pass β-carotene using Skellysolve F as a solvent. The Skellysolve should be free from ether, alcohol, or other solvents and be as dry as possible.

After the extract passes into the column, wash at once with Skellysolve F and continue washing until all carotene passes through the column. This is indicated by a colorless filtrate. This solution may be retained and the carotene measured.

The appearance of the column at this point shows a sharp colored layer at the extreme top, usually followed by a series of lines and a broad colored band, all in the carbonate layer. Remove all these with care, except for the lowest portion of the broad colored band, by digging out the adsorbent with a suitable instrument.

Change the receiving flask on the column and elute the vitamin A by passing 25 ml. of ether slowly through the column or until all the remaining yellow lines pass through the column. This eluate is to be used for the vitamin A reading.

Color Reaction. Divide the eluate from the column, or the original extract if the column purification was not required, into 2 equal portions of any convenient volume. At this point it is desirable to estimate the approximate vitamin A content of the material being tested, in order that the final solution concentration of vitamin A will be approximately 15 units/ml., a concentration most suitable for measurement. A volume factor, X, is approximated from the formula:

$$X = \frac{\text{(approximate units of vitamin A/g. of feed) (weight of sample)}}{30}$$

by which X is found to the nearest whole number (but not less than 2). To one portion of the solution add 10 x units of the vitamin A standard solution. Evaporate both fractions to dryness *in vacuo* and dissolve each fraction in exactly x ml. of dry chloroform. By this system one portion of the sample has been fortified with an internal standard to 10 units/ml.

The photoelectric colorimeter is set at 100% transmission with a reagent blank containing 1 ml. of chloroform plus 10 ml. of $SbCl_3$ reagent and the corresponding center setting (with reagent blank tube removed) is noted. This reading is used for subsequent settings for a series of readings on any one day with the same reagent.

Pipette 1 ml. of the unfortified unknown in a reaction tube in the instrument, add 10 ml. of $SbCl_3$ reagent, and take a reading as soon as the galvanometer can be read (reading A on Fig. 3). Place the reacted solution immediately in a glass-walled water bath (a rectangular battery jar is suitable) kept at 30° C. midway between two 150 watt reflector flood lamps kept at a distance of 30 cm. (12 in.) between the two bulb faces.

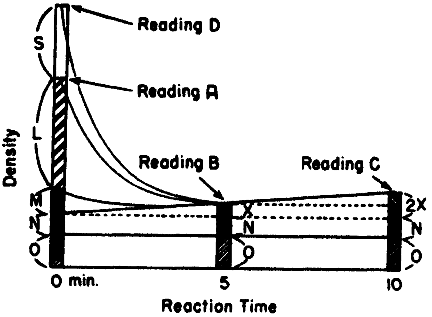


Fig. 3. Derivation of vitamin A calculation formula (after Brew and Scott (29)).

After exactly 5 min. read the reaction tube again (reading *B*) and immediately return it to the water bath. At the end of exactly 10 min. obtain a third reading (reading *C*).

React 1 ml. of the vitamin A-fortified sample as above with 10 ml. of $SbCl_3$ and take only the initial reading (reading *D*). This reading should not exceed a photometric density of 0.523 (corresponding to a transmission of 30%) in order to stay within the linear range for a vitamin A standard curve (*cf.* 105).

Dilute a second 1 ml. aliquot of the unknown (if necessary, a part of the fortified sample can be mixed with the unfortified for this reading) with 10 ml. of chloroform and read at 440 mμ (reading *E*) against a blank of pure chloroform set at 100% transmission. Reading *E* is made to correct for the interference of carotenoid pigments which give color reactions with $SbCl_3$. Calculate vitamin A concentration from the following formula in which all readings have been converted to photometric densities.

$$\text{Units of vitamin A/g.} = \frac{10(A - 2B + C - 0.067\ E)\ (2\ X)}{(D - A)\ (\text{weight of sample})}$$

The factor 0.067 in this formula is explained below, and should be determined for the particular type of carotenoid pigments most commonly encountered (in alfalfa, corn gluten, *etc.*), and with the particular filters used in making the readings.

d. Notes on the Procedure.

1) The method of assay for vitamin A in the presence of interfering

substances is shown in Fig. 3. Readings are made under controlled conditions of light exposure and temperature immediately after reaction (reading A), after 5 min. exposure (reading B), and after 10 min. exposure (reading C). In addition, a second portion of the extract is reacted in the presence of a known addition of standard vitamin A (reading D); an independent reading is made at 440 mμ (reading E) of the diluted but unreacted sample to be used as a correction for carotenoids present.

Reading A is composed of the sum of the light absorption produced by an unknown amount of vitamin A, represented by amount L, and three types of interfering colored reactions: materials which react to produce decreasing color intensities with time (amount M); materials which show increasing color intensities with time (amount N); and materials which react to produce a color that does not change with time during the short interval used or which have a color of their own which is unrelated to the $SbCl_3$ reaction (amount O). Reading B, at the end of 5 min., shows that L and M have faded out completely, O has remained constant, and N has increased by increment X. At the end of 10 min., represented by reading C, an additional increment, X, has been added to the value of original quantity N, making its value $N + 2X$.

$$\begin{aligned} \text{Reading } A &= L + M + N + O \\ \text{Reading } B &= X + N + O \\ \text{Reading } C &= 2X + N + O \end{aligned}$$

from which the formula, $L = A - 2B + C - M$, is derived. M may be derived by a separate reading (reading E) of the carotenoid color at 440 mμ which will bear a relationship, K, to the blue color produced at 620 mμ for any particular group of pigments and pair of filters.

Hence, $$M = KE$$
or $$L = A - 2B + C - KE$$

To compensate for the fact that certain materials may inhibit the amount of absorption L produced by the $SbCl_3$ reaction with vitamin A, a known increment (10 units = S) of "A" is added to a second fraction of the original solution and, after adjustment to the same volume for reaction, reading D is obtained.

All color produced by preformed vitamin A has completely faded at the end of 5 min. Thus, after this period of time, the sample with the added increment of vitamin A will read the same as the sample without the vitamin A increment (Fig. 3). This fact makes it possible to use the principle of the internal standard with its attendant advantages, as designed by Oser, Melnick and Pader (47).

$D - A$ equals the amount of absorption produced by 10 units of vitamin A under the same reaction conditions as in the case of L absorption, produced by the unknown amount of vitamin A. For low concentrations of vitamin A, the readings given in terms of photometric density are directly proportional to the amount of vitamin A present.

Therefore,

$$\frac{\text{Units of vitamin A}}{10} = \frac{L}{D - A}$$

or

$$\text{Units of vitamin A} = \frac{10\ (A - 2B + C - KE)}{D - A}$$

The value of K is derived by reacting with $SbCl_3$ and reading at 620 mμ portions of a series of solutions of varied carotenoid concentration, then reading dilutions of the same solutions at 440 mμ. The reacted absorption values are plotted against the dilution absorption values and a straight-line relationship is found. The ratio of the photometric density at 620 mμ to that at 440 mμ is the value of K desired. This was found to be 0.067 in the authors' colorimeter (29), for the carotenoids of alfalfa, the major source of carotenoids in feeds. This value was obtained after a chromatographic column treatment and readings obtained according to the procedure given. The chromatographic treatment removes pure carotene and only the related pigments are present.

2) The volumes and extraction times proposed in the procedure were determined for Butt-type extraction tubes. Other types of extraction equipment may require different conditions.

3) The authors obtained an average recovery of 93% of added vitamin A from mixed feeds.

C. VITAMIN A BY THE GLYCEROL DICHLOROHYDRIN METHOD

After Sobel and Werbin (71, 72)

a. Reagents.

Glycerol dichlorohydrin, practical grade of Eastman Kodak, or 1, 3-dichloro-2-hydroxypropane from Ohio Chemical Manufacturing Company, or a mixture of the 1, 3- and 2, 3-dichlorohydrins from the Shell Chemical Company.

Activated glycerol dichlorohydrin. To 1000 ml. of any of the above types of glycerol dichlorohydrin add 100 ml. of chloroform containing 10-50 g. of dissolved $SbCl_3$ (or the solid may be added directly). The mixture is distilled under a reduced pressure of 4-40 mm. of mercury in an all-glass apparatus. The chloroform fraction was discarded and the product collected and stored in a dry glass-stoppered bottle at room temperature. The dichlorohydrins from the Shell Chemical Co. were somewhat less satisfactory in the preparation of activated glycerol dichlorohydrin, because they required 2 distillations with $SbCl_3$ before a colorless reagent was obtained. The product thus obtained became colored in 3-4 weeks.

Chloroform, analytical reagent grade, dried over anhydrous Na_2SO_4, distilled, and kept over the same drying agent.

Antimony trichloride, reagent grade, Merck.

Standard Vitamin A Capsules, U.S.P.

b. Apparatus.

The authors (70-72) have used a *Beckman Model DU Quartz Spectrophotometer* with a tungsten light source and Corex cells of 1.00 cm. absorption path, as well as a *Coleman Universal Spectrophotometer,* Model 11, with a PC-4 filter. Preliminary trials have indicated that the *Evelyn photoelectric colorimeter* with filter 550 mμ may also be employed.

c. Procedure.

The extracts are prepared for colorimetry according to the procedure described under A. The final dilutions are made according to the instrument which is to be used for the measurement. On the Beckman spectrophotometer, a nearly linear relationship was observed at 555 mμ between 1.26 and 13.63 γ (5.7-61 U.S.P. units) of vitamin A. On the Coleman spectrophotometer, a nearly linear relationship existed at 550 mμ between vitamin concentration and optical density in a range of 2-5.5 γ (9-25 U.S.P. units) of vitamin A. In the Evelyn colorimeter, we found the range for linearity to be 2-11 γ (10-50 U.S.P. units) of vitamin A.

All dilutions are made, and all colors developed, in a constant temperature bath at 25° C. For the Beckman and Coleman instruments, 1 ml. of chloroform containing the desired concentration of vitamin A, or carotene, or both, is added to 4.0 ml. of activated glycerol dichlorohydrin in a 10 ml. glass-stoppered cylinder. For the Evelyn colorimeter, the amounts used are 2.0 ml. of extract and 8.0 ml. of reagent (the instrument being set with 2.0 ml. chloroform + 8 ml. reagent) and the reaction carried out directly in Evelyn test tubes. The mixture is shaken several times to ensure homogeneity and placed in a water bath until read either spectrophotometrically or colorimetrically.

A calibration constant is obtained by establishing the relationship between vitamin A concentration and optical density with the use of standard vitamin A capsules. The establishment of the calibration constant is described in detail under A. The calculation for the Evelyn colorimeter is carried out as usual, *i.e.,*

$$\frac{L}{K} \times \text{Dilution factor} = \text{U.S.P. units of vitamin A/sample.}$$

d. Notes on the Procedure.

1. Under the conditions employed for vitamin A determinations with activated glycerol dichlorohydrin, interference by vitamin D and related sterols is negligible.

2. Interference by carotene can be evaluated by the method of Dann and Evelyn (see under A), or by reading at 555 and 800 mμ in the Beckman spectrophotometer, since the violet vitamin A color does not absorb at 800 mμ.

D. VITAMIN A AND CAROTENE IN WHOLE DRIED EGGS

After Thompson, Ewan, Hauge, Bohren and Quackenbush (24)

a. Reagents.

Methanol, absolute, may be used without purification.

Potassium hydroxide, saturated, aqueous (from C.P. KOH).

Ethyl ether, peroxide-free. Anhydrous ethyl ether (alcohol-free) was allowed to stand several days over KOH and then redistilled shortly before use.

Sodium sulfate, C.P., anhydrous, "Special for Nitrogen Determination."

Petroleum ether, dry, treated as follows before use: 4-5 gal. of commercial petroleum ether (B.P. 65-67° C.) were percolated through a column (7 × 35 cm.) of silica gel (The Davidson Chemical Corp., No. 659-528-2000). The percolate was then placed in a 5 gal. flask and stirred mechanically for several hr. with each of 2 successive portions of concentrated H_2SO_4. The 2 phases were separated and the last trace of acid was removed by treatment with a dilute solution of NaOH. The petroleum ether was then stirred for a few hours with alkaline $KMnO_4$, after which it was drawn off, distilled, and dried over anhydrous sodium sulfate.

Chromatographic adsorbent. Three parts of $Ca(OH)_2$ (Braun's Lot No. 10,588) and two parts of Hyflo Super-Cel.

Eluting solution. 60% benzene (thiophene-free) and 40% dry petroleum ether.

Chloroform, purified as follows: chloroform (A.R.) was distilled over anhydrous K_2CO_3 and stored in a brown bottle.

Antimony trichloride reagent. 22.5 g. of $SbCl_3$ (C.P.) were dissolved in 100 ml. of purified chloroform; the reagent was filtered and stored in a brown bottle.

Standards. For vitamin A standard, see description in section 2A, a). For the β-carotene standard, a good sample of β-carotene is purified by chromatography.

b. Apparatus.

A *direct-reading photoelectric colorimeter* with interchangeable test tubes and 440 and 620 mμ filters. The direct-reading galvanometer must be critically damped, and have a short period.

A 9 ml. automatic pipet. Delivery should require not more than 2 sec. (*cf.* Fig. 2).

Chromatographic column. 20 × 135 mm.

c. Procedure.

A 5.0 g. sample is weighed into a 125 ml. Erlenmeyer flask and 20 ml. of absolute methanol and 5 ml. of saturated aqueous KOH are added. The contents are stirred with a glass rod until complete suspension of the sample is effected. The flask is then heated on a steam bath for 10 min., or until all solid particles are disintegrated. The hydrolyzate is then cooled and transferred with 70 ml. of water (in three portions of 30, 20, 20 ml.) to a 500 ml. separatory funnel. The first extraction is made with 35 ml. of peroxide-free ethyl ether and the four subsequent extractions with 25-30 ml. portions. The last extractions should be almost colorless. The pooled ether extract is washed 5 times with 25 ml. portions of water, then dried over 20 g. of anhydrous Na_2SO_4 for 1 hr. at room temperature. The filtered ether extract is evaporated to approximately 15 ml. under reduced pressure in a 50° C. water bath and then transferred to a 25 ml. volumetric flask with dry petroleum ether. If the solution still contains some moisture, as indicated by cloudiness, it should be dried with a small amount of Na_2SO_4.

In the chromatographic separation, 10 ml. of this solution are adsorbed on a column filled with the adsorbent. The chromatogram is developed with the eluting solution. The two lowest bands, containing β-carotene and cryptoxanthol, are eluted separately. The vitamin A fraction is collected until the luteol begins to give a yellow color to the eluate. If the column is properly packed, the β-carotene and cryptoxanthol bands are easily distinguished. The luteol band should be sharp as it nears the bottom of the column. Fifty to 80 I.U. of vitamin A and 150-200 γ of total carotenoids (as β-carotene) can be handled satisfactorily with this column. A total volume of 250-500 ml. of combined β-carotene, cryptoxanthol, and vitamin A eluate gives the optimum dilution.

After removing the solvent from the β-carotene and cryptoxanthol fractions, these are taken up in 10 ml. of petroleum ether and the yellow color measured at 440 mμ. These solutions are then recombined with the vitamin A fraction and the solution is evaporated under reduced pressure at 50° C. to approximately 15 ml. This solution is then transferred to a 25 ml. volumetric flask with redistilled chloroform. Five ml. of this solution are evaporated to dryness in a colorimeter tube and the residue is taken up in 1.0 ml. of chloroform. The tube is placed in the colorimeter, 9.0 ml. of $SbCl_3$ reagent are added and the blue color measured at 620 mμ within 5-10 sec.

This total blue color is corrected for the presence of β-carotene and cryptoxanthol by reference to a previously prepared correction curve relating the blue color of these two compounds with their yellow color as measured at 440 mμ. For this purpose, as well as for the calculation of the concentration of cryptoxanthol, this provitamin A is considered equivalent to β-carotene.

E. CAROTENE IN ALFALFA, SWEET POTATOES, AND CARROTS

After Kemmerer (102)

a. Reagents.

Alcoholic potassium hydroxide. Dissolve 12 g. of KOH in 100 ml. of 95% ethyl alcohol.

Petroleum ether. B. P. 30-70° C. or Skellysolve.

Adsorbent. Use $MgCO_3$, $CaHPO_4$, or other adsorbents which will adsorb less than 5% carotene when tested by the following method. Place approximately 2 g. of the adsorbent in a tube as described below and pass through it 50 ml. of a solution of purified carotene in petroleum ether containing 1.0-1.5 γ of carotene/ml. Wash the column with petroleum ether and determine the carotene in the filtrate. If the adsorbent is too retentive of carotene, another lot should be tried. If, later in the analyses, the adsorbent is found not to have sufficient adsorptive power to separate the xanthophylls and other impurities from the carotene mix with it a small amount of MgO (*ca.* 2.5%). The amount of MgO will depend on the adsorptive power of the carbonate and of the oxide.

Purified carotene solution. Dissolve about 0.1 g. crystalline carotene (90% β, 10% α) in about 2-3 ml. of chloroform. Add 25 ml. methanol and filter. Wash the crystals of carotene once with a few ml. methanol. Dry in a vacuum over anhydrous $CaCl_2$ for not more than 1 hr. Dissolve 20 mg. of the purified carotene in about 2 ml. of chloroform and dilute to 100 ml. with petroleum ether. This stock solution contains 200 γ/ml. of carotene.

b. Apparatus.

A photoelectric colorimeter with 440 mμ filter.

c. Procedure.

(1) Extraction of Materials (Dried Hays, Grasses, etc.) Other than Yellow Corn, Sweet Potatoes, and Carrots.

Reflux a 1-5 g. sample for 30 min. with 20-100 ml. of 12% alcoholic KOH. If portions of the sample collect on the sides of the flask, wash

down with alcohol. After refluxing, cool the contents of the flask, add 50 ml. of petroleum ether, shake for a minute, and allow the sediment to settle. Decant the liquid into a 500 ml. separatory funnel. Repeat extraction twice more with 25 ml. portions of petroleum ether. Break up the residue, which sometimes forms an adherent mass, by shaking with 10-15 ml. of 95% ethyl alcohol. Make 2 or 3 additional extractions with 25 ml. portions of petroleum ether, and continue until the petroleum ether is colorless.

Pour gently about 100 ml. of distilled water through the alcohol-petroleum ether solution into a separatory funnel. Draw off the alkaline alcohol-water solution and extract once more by shaking with 30 ml. of petroleum ether in another funnel. Any emulsion may be cleared by adding 1 ml. of ethyl alcohol. Combine the petroleum ether extracts and wash these 5 times with 50 ml. portions of distilled water. Concentrate, if necessary, filter over anhydrous Na_2SO_4, and make up to a volume of 100 or 200 ml.

(2) Extraction of Dehydrated Carrots and Sweet Potatoes.

To 1 or 2 g. of finely ground sample add approximately 4 or 8 ml. of distilled water and allow to stand 30 min. If the dehydrated material contains considerable moisture, and is consequently too tough to grind in a Wiley or other type of mill, soak 5 g. of the unground material in 20 ml. of water for 2 hr. Then grind in a mortar with a pestle.

Add 50-100 ml. of 95% ethanol and reflux 15 min. Filter the alcoholic solution through a fritted glass funnel and wash the residue with small portions of ethanol. Remove the residue, add 25 ml. of 95% ethanol, and reflux again for 15 min. Filter and wash as above. If the residue contains more than a very slight amount of yellow color, reflux a third time. To the combined alcoholic solutions and washings, add 50-100 ml. petroleum ether. Shake, and then add 100-150 ml. of water and allow the layers to separate. Draw off the aqueous-alcoholic layer and reextract with 25-50 ml. of petroleum ether. Repeat this extraction until the petroleum ether layer separates colorless, or only very slightly colored. Combine the petroleum ether extracts and wash 5 times with 50 ml. portions of distilled water. Concentrate if necessary, filter over anhydrous Na_2SO_4 and make up to a volume of 100 or 200 ml.

Purification of Pigments. Prepare a column as follows. Place about 2 g. of adsorbent in a glass tube about 10 mm. wide and about 15 cm. tall, constricted at one end and plugged with a wad of cotton. Apply suction and pack the $MgCO_3$ firmly, but not too tightly, with a cork having a smooth surface and attached to a glass rod. Concentrate to about 10 ml., *in vacuo,* a 50 ml. aliquot of the carotene solution obtained above. Place a few ml. of petroleum ether on the column of adsorbent, apply suction, and, before the petroleum ether is drawn in, place the concentrated aliquot in the tube. Allow all the extract to be very nearly drawn into the column and then add petroleum ether. Always keep the surface of the column covered with petroleum ether. Wash the column with petroleum ether until the pure carotene is washed through. Collect in a suitable container and dilute to 50 ml.

Determination of Carotene in Extracts. The photoelectric colorimeter with the 440 mμ filter must first be calibrated against pure carotene. Purify crystalline carotene as given above, and make up a stock solution containing 200 γ/ml. of carotene. From this solution, make up solutions containing 2.0, 1.6, 1.2, 0.8, 0.4, and 0.2 γ/ml., and obtain the colorimeter readings for these carotene solutions. Plot colorimeter readings against concentration of carotene on arithmetic graph paper. Use this curve for estimating carotene in the sample. Photoelectric colorimeters may vary in their sensitivity and are checked by means of a $K_2Cr_2O_7$ solution containing 0.02% $K_2Cr_2O_7$. Instruments are adjusted always to give the same reading for this solution. If the instrument cannot be adjusted to give the desired reading, recalibrate the instrument.

After the photoelectric colorimeter has been calibrated, read the color of the carotene solutions and calculate γ of carotene/g. of sample.

A spectrophotometer may also be used to measure the carotene color.

F. VITAMIN A AND CAROTENE IN BUTTER

After Berl and Peterson (9)

a. Reagents.

Potassium hydroxide, solid (C.P.).

Methanol, aldehyde-free.

Diethyl ether, peroxide-free.

Skellysolve, B.

Diacetone-alcohol, 94% by volume.

Antimony trichloride reagent, 20% $SbCl_3$ in chloroform.

b. Apparatus.

A *direct-reading photoelectric colorimeter* with filters at 440 mμ and 620 mμ. The authors (9) used an Evelyn photoelectric colorimeter.

c. Procedure.

Whole butter may be used if the fat content is known. If not, the butter fat is obtained by melting the butter on a water bath below 60° C. and filtering it through cotton. It is then allowed to solidify in the refrigerator. The cake is removed and scraped free of any adhering particles of moisture and non-fat solids. Ten g. of fat are weighed into a suitable saponification flask. Five g. of solid KOH, 5 cc. of water, and 20 cc. of aldehyde-free methanol are added, and the flask is connected by a ground glass joint to a condenser and refluxed on a water bath for 10 min. The sample is then diluted with 40 cc. of distilled water, transferred to a separatory funnel, and the flask rinsed with an additional 40 cc. of water. The mixture is extracted with one 100 cc. and two 50 cc. portions of peroxide-free ether. The combined extracts are washed with 100 cc. portions of distilled water until the washings are free of alkali. Four washings should be sufficient. The ether solution is dried over anhydrous Na_2SO_4 and transferred to a round-bottom Claisen flask; the Na_2SO_4 is washed 3 times with 10 cc. portions of ether and the washings added to the main solution. Several glass beads are added to prevent bumping. The flask is placed on a warm water bath and the ether removed under reduced pressure. Care must be taken not to continue heating the flask after the ether has been removed. About 15 cc. of Skellysolve B is added before the vacuum is released. If the flask is still warm, it is cooled under the water tap. The solution and rinsings of the flask with Skellysolve B are transferred to a 50 cc. volumetric flask and made up to volume.

A 5-cc. aliquot is diluted to 10 cc. and read for total pigment. Another aliquot of 25 cc. is extracted with three 10 cc. portions of 94% diacetone alcohol to remove noncarotene pigments, washed once with 10 cc. of water, made up to 50 cc. with Skellysolve B and read for carotene.

For the vitamin A determination, 1 cc. of the original Skellysolve B solution is placed in an Evelyn tube, the Skellysolve B is removed under vacuum and replaced by 1 cc. of chloroform. Nine cc. of the $SbCl_3$ reagent are added from a rapid-delivery pipette. The galvanometer is read at the point of temporary stability.

To correct this blue color reading for the color produced by carotene and other pigments, Berl and Peterson (9) use the following equation:

L_{620} (corrected) $= L_{620}$ (observed) $-$ ($0.007 \times \gamma$ total pigment expressed as carotene).

d. Notes on the Procedure.

1) The factor 0.007 was obtained by establishing the relationship between the amount of blue color formed with $SbCl_3$ and the intensity of the yellow color of the pigments. Each laboratory should establish its own factor by studying this relationship under the particular experimental conditions used.

2) The volumes given are suitable for summer butters, but for winter butters, which have less pigment, a greater concentration of the final solution may be required.

3) Results obtained by this method were in very good agreement with concurrent chromatographic assays.

G. RAPID METHOD FOR CAROTENE IN VEGETABLE OILS

After Bickoff and Williams (13)

a. Reagents.

Aluminum oxide, 80-mesh, activated especially for chromatographic analysis, manufactured by the Aluminum Ore Company, East St. Louis, Ill.

Petroleum ether, B.P. 30-60° C.

Eluting solution, petroleum ether containing 2% acetone.

Carotene standard, crystalline, 90% β- and 10% α-carotene.

Sodium sulfate, anhydrous.

b. Apparatus.

A *photoelectric colorimeter* with filter at 440 mμ. The authors (13) used an Evelyn photoelectric colorimeter.

c. Procedure.

The columns are prepared in the following manner. Tswett tubes having an inside diameter of 11 cm. are packed with 12 g. of Al_2O_3. Columns of uniform height of about 10 cm. are obtained by applying gentle suction and tamping lightly with a heavy glass rod spread and flattened on one end.

One g. of the vegetable oil solution of carotene is weighed into a 100 ml. volumetric flask and made up to volume with petroleum ether. A 10 ml. aliquot of this solution is run onto the column. After all the solution has passed into the Al_2O_3, the column is developed and the carotene is simultaneously eluted with the required amount of petroleum ether containing 2% acetone (no pressure or vacuum is required to hasten the percolation).

The volume of eluant needed must be determined for each batch of Al_2O_3. As the commercial preparation of Al_2O_3 may vary somewhat in activity from one lot to another, a preliminary determination of the volume of eluant is required. The required calibration may be readily made as follows. A 10 ml. aliquot of a petroleum ether solution of crystalline carotene, containing 0.1 g. of oil (without added carotene)/ml. of solution, is run onto the column. Several columns are prepared in this manner. Columns are then washed with various amounts of 2% acetone in petroleum ether to determine the minimum amount of eluant needed to remove all the carotene. This volume is then used in the analyses of carotene-enriched vegetable oils for eluting the carotene and leaving the other colored impurities on the column.

The carotene eluate is caught in a 100 ml. volumetric flask and made to volume, and the carotene concentration is determined colorimetrically in the Evelyn photoelectric colorimeter with the 440 mμ filter. This measures β-carotene, together with any α- or neo-β-carotene that may be present. Both α- and neo-β-carotene are more readily eluted from the Al_2O_3 column than β-carotene.

d. Notes on the Procedure.

1) The importance of testing any particular batch of Al_2O_3 to determine the volume of eluant needed to obtain quantitative recoveries of carotene is emphasized by the data of Bickoff and Williams (13); these authors found that the columns prepared from the first three 5-lb. lots of Al_2O_3 required 45 ml. of eluant to remove the carotene, while the columns prepared from the fourth 5-lb. lot required 65 ml. of eluant.

2) The Al_2O_3 must be protected against adsorption of moisture to keep its activity constant.

3) The method can also be used to separate carotene from other pigments in a petroleum ether solution that is free of oil. More eluant is needed to remove carotene from the column when no oil is present. The volume of eluant required may be readily determined as described above by use of a petroleum ether solution of carotene containing no oil. The volume of eluant required for columns prepared from various lots of Al_2O_3 ranged from 75 to 90 ml.

H. RAPID EXTRACTION PROCEDURE IN THE DETERMINATION OF VITAMIN A AND CAROTENE IN MILK

After Boyer, Spitzer, Jensen, and Phillips (45)

a. Reagents.

Potassium hydroxide solution. Add 10 ml. of distilled water to 20 g. of KOH (U.S.P. pellets), mix until dissolved, and shake with 90 ml. of absolute ethyl alcohol.

Antimony trichloride solution. Rapidly weigh and transfer 20 g. of $SbCl_3$, reagent grade, to a brown glass-stoppered bottle and add 100 ml. of chloroform (U.S.P. grade or better). Solution may be hastened by breaking up the lumps with a stirring rod. Filter on a rapid qualitative paper before use.

Acidified alcoholic wash solution. Add 1 ml. of HCl, C.P., to 100 ml. of ethyl alcohol and make up to 1 liter.

Petroleum ether, Skellysolve B.

Diethyl ether, U.S.P. grade or better; for exact work peroxide-free ether should be used.

Chloroform, C.P.

Absolute ethyl alcohol, aldehyde-free.

Sodium sulfate, anhydrous, reagent grade.

Acetic anhydride, reagent grade.

b. Apparatus.

A *direct-reading photoelectric colorimeter* with filters at 440 and 620 mμ. The authors (45) use the Evelyn photoelectric colorimeter.

Rapid-delivery pipet (9 ml.). A convenient, rapid-delivery pipet may be constructed by cutting off a 10-ml. pipet, constricting the tip to 2.5 mm. inside diameter, and recalibrating for the rapid delivery of 9.0 ml. The pipet is joined to a three-way stopcock for regulation of filling and delivery and is attached to a rubber suction bulb.

Pyrex test tubes, 75 ml.

Y-tubes, fitted with rubber stoppers for evaporation of solvents.

c. Procedure.

(1). Extraction. Thirty ml. of the alcoholic KOH solution are added to a 20.0 ml. sample of fresh, whole milk in a separatory funnel. The contents are mixed by brief, vigorous shaking, and allowed to stand for 3 hr. A yellow coloration which develops does not interfere. Then 25 ml. of diethyl ether are added and the separatory funnel is stoppered tightly and shaken vigorously for 1 min. After separation, the lower layer is drawn off into a second separatory funnel. Any small amount of emulsified material is retained. The residue in the second funnel is extracted by shaking vigorously for 1 min. with 18-20 ml. of ether. Vapors formed are allowed to escape by carefully opening the stopcock of the inverted funnel. After separation, the lower layer is discarded.

To the first separatory funnel 75 ml. of distilled water are added and mixed with the ether extract by inverting the funnel once. The water layer is drawn off into the second separatory funnel, extracted by shaking vigorously for 1 min. with the ether already present, then discarded. The ether extract in the first funnel is washed by shaking briefly with about 10 ml. of the acidified alcoholic wash solution. The wash mixture is drawn off and shaken with the ether in the second funnel, then discarded. Three ml. of petroleum ether are added to each funnel to reduce the water content. The extract in the first funnel is washed twice more with 10 ml. portions of the wash mixture and the washings are extracted singly in the second funnel, then combined and allowed to stand 15-20 min. Any water settling out is carefully removed.

The extract is then transferred to a 75 ml. Pyrex test tube. The solvents are removed by attaching two such tubes to a vacuum pump (equipped with a water trap) by means of a Y-tube. The vacuum is slowly increased with continual gentle rotatory shaking of the inclined tubes in a water bath at 30-40° C. until it is possible to apply full vacuum without excessive frothing of the sample. Then the tubes are heated with continual shaking in a bath at 60-70° C. until all solvents are removed, but no longer. The tubes are cooled in a beaker of cold water, the vacuum is released, and 5.0 ml. of ether (accurately measured) are added. The residue is dissolved and shaken with 5 ml. of saturated NaCl solution, then 10.0 ml. of petroleum ether (accurately measured) are added, and the contents are shaken vigorously. After standing for several min., the extracts should be crystal clear. If not, the tubes need to be shaken again.

(2). Analysis. A 10.0 ml. aliquot is transferred to an Evelyn colorimeter tube, and the total carotenoids are measured using the 440 mμ filter. Then the colorimeter tube is attached to a vacuum pump, and the solvent is evaporated as before. The residue is dissolved in 1.0 ml. of chloroform. A drop of acetic anhydride is added to remove any traces of water. The tube is placed in the colorimeter and 9.0 ml. of the $SbCl_3$ reagent are added from a rapid delivery pipet. The maximum deflection which occurs using the 620 mμ filter is measured.

The calculations and expression of results may be made according to the method outlined in the first method presented in this section (*cf.* Dann and Evelyn (42)). However, the authors of this method (45) prefer to express the results in terms of micrograms on the basis of the following calibration of the colorimeter. The carotene readings are compared to a standard curve prepared by readings with solutions of crystalline carotene. However, for routine work a constant may be used for the calculation of carotene if the galvanometer readings are 35 or above. The authors (45) found this constant to be 28, so that

$$L_{440} \times 28 = \gamma \text{ of carotene/10 ml. of Skellysolve B.}$$

The blue color produced by the Carr-Price reaction follows Beer's law and a constant is evaluated by standardizing the colorimeter with pure vitamin A, or less preferably, with a standardized fish oil. The constant used by the authors (45) was 13.2, so that

$$L_{620} \times 13.2 = \gamma \text{ of vitamin A/10.0 ml. of chloroform.}$$

For the calculations, the vitamin A reading is corrected for the Carr-Price reaction of the carotenoids, using the correction factor of 0.14 (42). The calculation is:

$(L_{620} - 0.14\ L_{440}) \times 13.2 = \gamma$ of vitamin A/10.0 ml. of chloroform. For the dilutions used in the above procedure the γ of carotene or vitamin A/10.0 ml. of solvent $\times$ 7.5 = γ/100 ml. of whole milk.

d. Note on the Procedure.

The carotene determination, as given in the procedure, is a measure of the total carotenoids in the milk. The carotenoids in cow's milk have been shown to be principally β-carotene (15).

II. Vitamin D

The most satisfactory and most specific method of vitamin D determination is the animal experiment. Numerous attempts have been made to use physicochemical methods such as (a) direct absorption spectroscopy and (b) colorimetry of reaction products for the estimation of vitamin D. The weak and only moderately characteristic absorption of vitamin D in the ultraviolet region militates against direct spectroscopy as a possible analytical procedure. On the other hand, the colorimetric methods are hampered by their reduced specificity and by interference of coexisting substances.

Direct colorimetry without further purification of the sample in question may be applicable only to the most highly concentrated materials such as irradiated ergosterol, irradiated 7-dehydrocholesterol, and purified fish oil extracts. Under such circumstances the colorimetric method of Brockmann and Chen (106) might be of practical usefulness.

The method of Brockmann and Chen (106) is based on the production of an orange color when a dry, alcohol-free solution of $SbCl_3$ (in chloroform) is added to a solution of vitamin D_2 (or D_3). The maximum of absorption is at 500 $m\mu$ and may be read in a colorimeter with the use of the appropriate filter, or in a spectrophotometer (Coleman or Beckman). Tachysterol gives the same color reaction as vitamin D_2 (or D_3). Sterols (cholesterol) may be present without interfering with the reaction up to a concentration 80 times that of vitamin D in the solution to be tested. Vitamin A gives the Carr-Price reaction with a very different maximum of absorption (at 620 $m\mu$) and consequently will not interfere with the determination of vitamin D, provided its concentration is not greater than about 6 times that of vitamin D present.

Reagent. Cold saturated solution of pure dry $SbCl_3$ in dry chloroform (free from even a trace of alcohol). Chloroform should be purified by distillation and should give a negative test with KI and starch. The solution of $SbCl_3$ in chloroform should stand at least 24 hr. before its use and be renewed at weekly intervals.

Apparatus. Photocolorimeter or Spectrophotometer. A standard curve is established for the photocolorimeter in the conventional manner using various solutions of pure vitamin D_2 (or D_3).

The color is developed by adding 4 (or 5) cc. of the $SbCl_3$ reagent to 0.2 (or 0.25) cc. of the test substance (or to its solution in chloroform).

The maximum of color is reached in about 10 min., with best reading between 10-15 min.

The sensitivity of the reaction is not greater than 20 γ of vitamin D with the upper limit of about 400 γ.

Removal of interfering substances by chromatographic adsorption will increase the sensitivity of the colorimetric determination of vitamin D (107).

The schematic outline of the method proposed by Ewing *et al.* (107) is given in stages I to V (See Table II). The authors themselves concede that "the procedure is somewhat complicated; in fact, the minutest details should be followed exactly." In consequence, the instructions of the authors should be given in detail.

Equipment and Reagents.

"1. The adsorption columns must be carefully and uniformly prepared to obtain reproducible results. The tubes for the chromatographic determinations are made by sealing a 6 cm. length of 7 mm. Pyrex tubing to the bottom of a 1.6 × 15 cm. (0.625 × 6 in.) Pyrex test tube. These tubes are cleaned before filling by soaking in sulfuric acid-dichromic acid solution, rinsing with distilled water and with alcohol, and finally drying in the flame of a Meker burner. The suction apparatus is designed so that a bank of 8 columns can be developed simultaneously, controlling the pressure with the aid of an open-tube mercury manometer attached to the suction flask. The adsorbent used is a finely divided grade of activated bentonite clay (Superfiltrol, obtained from The Filtrol Corp., 315 W. 5th St., Los Angeles, Calif.).

"The adsorption columns for the first chromatographic separation are prepared by placing a small wad of cotton in the bottom of one of the adsorption tubes, pressing this down firmly, and adding enough of the clay, so that when very firmly pressed down with a piston, using a cork on the end of a glass rod, under 6 cm. of suction, the height of the packed column will be 3 cm. A second and equal portion of the adsorbent is then added, and pressed down as before to give a hard, level surface. It is important that there should be no air pockets, as they cause irregularly shaped adsorption bands. To aid in overcoming this, the piston-head cork used in packing the columns is but slightly smaller than the inside diameter of the Pyrex tube. This also helps avoid loosening of the adsorbent by suction when this cork piston is raised. Other columns for the chromatographic separation of vitamins D and sterols are prepared in the same manner, except that the height is only 1.5 cm. and the filling is done under a suction of 4 cm.

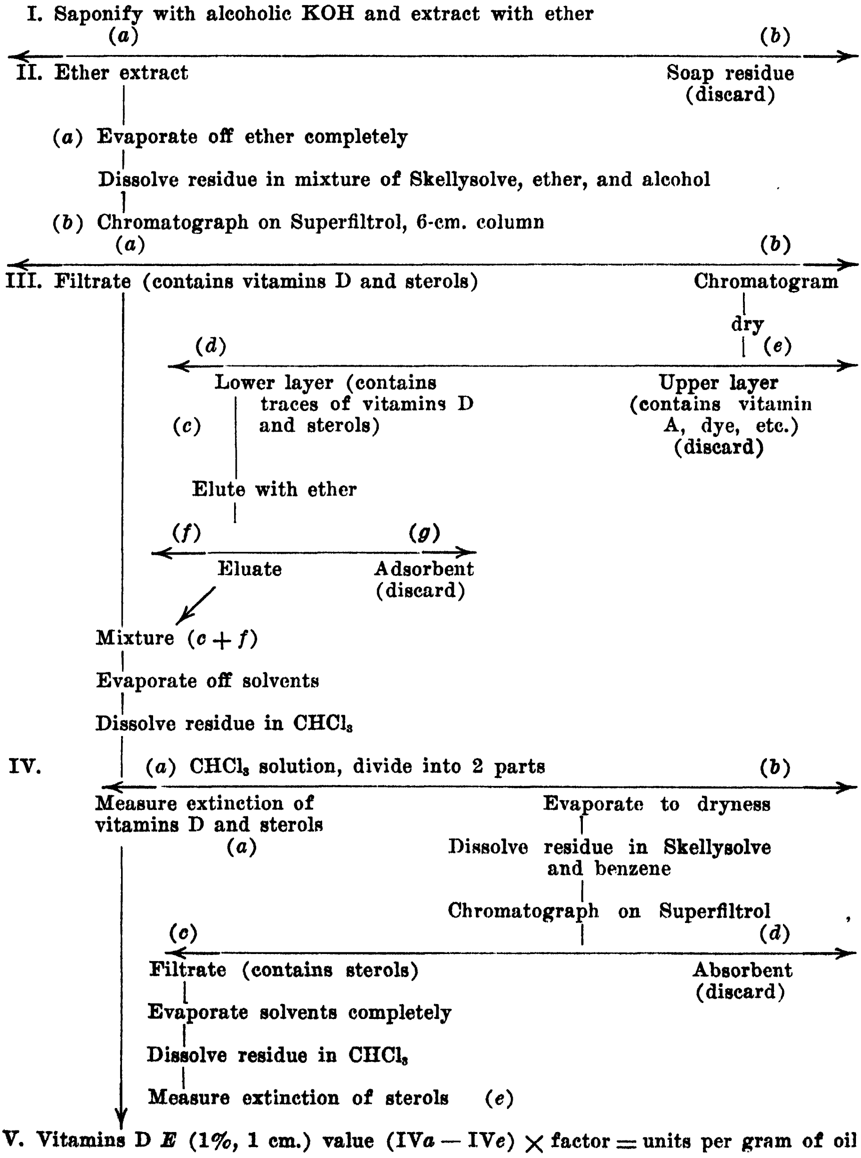
"2. Alcoholic KOH is prepared by dissolving 14 g. of C.P. KOH pellets in 95% ethyl alcohol to give 500 ml. This stock solution is protected from CO_2 and filtered through hardened filter paper as needed.

"3. C. P. ethyl ether is used without further purification for extracting the saponified oils and for elution of adsorption columns.

"4. The anhydrous ethyl ether for the chromatograph is purified by

TABLE II

Schematic Outline of Vitamins D Determination


I. Saponify with alcoholic KOH and extract with ether
(a)
(b)
II. Ether extract
Soap residue (discard)
(a) Evaporate off ether completely
Dissolve residue in mixture of Skellysolve, ether, and alcohol
(b) Chromatograph on Superfiltrol, 6-cm. column
(a)
(b)
III. Filtrate (contains vitamins D and sterols)
Chromatogram
dry
(d)
(e)
Lower layer (contains traces of vitamins D and sterols)
Upper layer (contains vitamin A, dye, etc.) (discard)
(c)
Elute with ether
(f)
(g)
Eluate
Adsorbent (discard)
Mixture (c + f)
Evaporate off solvents
Dissolve residue in CHCl₃
IV.
(a) CHCl₃ solution, divide into 2 parts
(b)
Measure extinction of vitamins D and sterols
(a)
Evaporate to dryness
Dissolve residue in Skellysolve and benzene
Chromatograph on Superfiltrol
(c)
(d)
Filtrate (contains sterols)
Absorbent (discard)
Evaporate solvents completely
Dissolve residue in CHCl₃
Measure extinction of sterols
(e)
V. Vitamins D E (1%, 1 cm.) value (IVa — IVe) × factor = units per gram of oil

washing C. P. ether with 1% $FeSO_4$ solution to remove peroxides, then 10 times with distilled water to remove alcohol, drying with P_2O_5, filtering, and storing over sodium. This ether is distilled from metallic sodium as needed.

"5. The Skellysolve is purified by shaking with concentrated H_2SO_4, washing twice with 10% Na_2CO_3 solution, then with a mixture of 10% Na_2CO_3 and 5% $KMnO_4$ solution. It is washed 15 times with distilled water, the reagent being decanted into a dry flask and dried over sodium. The dried solvent is then distilled (68-70° C.) from sodium, the first 5% and the last 10% of the distillate being discarded.

"6. The absolute ethyl alcohol is a high-grade commercial product.

"7. C. P. chloroform is washed thoroughly with 7 approximately equal portions of water, dried over anhydrous K_2CO_3, decanted, and fractionated, discarding the first and last 10% of the distillate. This purified chloroform is relatively unstable and hence is prepared in small quantities, and protected from light. Before use, it is tested with $AgNO_3$ solution for chlorides and with KI and starch solution for oxidizing agents. If these are absent, the chloroform is shaken with activated carbon and filtered just before using.

"8. The $SbCl_3$ reagent is prepared by dissolving 18 g. of C. P. $SbCl_3$ in the purified chloroform, diluting to 100 ml., filtering, and then adding 2 ml. of redistilled acetyl chloride (108). The best results are obtained when not more than one week's supply of the purified chloroform and $SbCl_3$ reagent is prepared at one time.

"9. C. P. thiophene-free benzene is dried over sodium, distilled, and shaken with Superfiltrol before use."

10. Measurements of the absorption maxima at 500 mμ are made with the use of a Bausch & Lomb, Beckman, or any similar spectrophotometer.

Application of Method.

"Fish oil samples are weighed in duplicate into 125 ml. Erlenmeyer flasks. Samples containing 4,000-100,000 U.S.P. units of vitamins D are most convenient. Usually, 0.5-2 g. are weighed for natural oils and 0.1 g. for concentrates. If the samples weigh 1 g. or less, 10 ml. of alcoholic KOH are added, but for samples weighing more than 1 g., 10 ml. of alcoholic KOH/g. of sample are used. A short-stemmed funnel is then placed in the neck of the flask, to serve as a condenser. The sample is placed in a water bath and kept at 70-75° C. for 1 hr., or longer if saponification is not complete. Agitation of the flask at frequent intervals aids this reaction. (At this point the sample may be allowed to stand overnight.)

"The sample is cooled to room temperature, 20 ml. of water/10 ml. of alcoholic KOH are added, and it is then extracted in a separatory funnel with four 25 ml. portions of ethyl ether. Gentle shaking in the separatory funnel can be used without danger of emulsification, provided more than the indicated quantity of water has not been added. If an emulsion does form, it can be readily broken by the addition of a few drops of alcohol. The ether layer must always be clear before separation of the two layers.

"Finally, all the ether extracts are combined in the separatory funnel and 50 ml. of water are added. When both ether and water layers are clear, the water layer is withdrawn and discarded, leaving the ether layer in the funnel. This preliminary washing, which removes most of the soaps, is repeated twice with fresh 50 ml. portions of water. Agitation during these preliminary washings may result in the formation of a stable emulsion. If this occurs, 2 ml. of alcohol are added to break the emulsion. Twenty-five ml. of water are now added to the ether extract in a separatory funnel and shaken vigorously, after which 25 ml. of water are added and the water and ether layers are allowed to separate. The water layer must be clear before it is withdrawn. This is again repeated twice; at the end of the third washing the ether and the water layers should separate quickly to a sharp interface and appear very clear. The water layer at this point should be colorless and not alkaline to phenolphthalein. If not, the washing must be continued until these conditions are satisfied.

"The washed ether extract is now withdrawn into a 125 ml. Erlenmeyer flask, passing through a filter paper containing anhydrous Na_2SO_4. The separatory funnel is rinsed with 25 ml. of ether and the rinsings are poured on the filter to remove vitamins D from the Na_2SO_4 and filter paper. These should be colorless. The ether solution is then evaporated to dryness under reduced pressure, using a water bath at 50° C.

"The dry residue from this evaporation is taken up in 5 ml. of a special mixture of solvents prepared from the above purified materials—50 parts of Skellysolve, 10 parts of anhydrous ether, and 1 part of absolute alcohol by volume. One drop of Sudan III solution (25 mg./l. in the above mixed solvents) is added as a color marker in the separation of vitamin A and pigments from vitamins D.

"The 6 cm. adsorption column (prepared as previously described) is wetted with 10 ml. of the mixed solvent, and the sample is added, followed by 5 ml. of the solvent for rinsing the flask and 35 ml. of the same mixture for developing the adsorption bands. Each addition of solvent is made just before the top of the adsorption column becomes dry, otherwise it shrinks away from the glass walls and development of the column is not regular. A pressure differential of 6 cm. of mercury is maintained until 35 ml. of the developing solution have been added, when the suction is increased to 10 cm. Further increase of the suction results in crevices in the adsorption column and packing of the adsorbent to such a degree that the flow of the developing solution is nearly stopped.

"When the last of the 35 ml. of solution has passed through the column, the adsorbent is dried by drawing air through the column for 5-10 min. The Pyrex tube is then taken from its suction flask and the top layer of the column is removed to a point 2 mm. below the red Sudan III band, using an L-bent spatula. This removed the vitamin A and pigments. The tube with the remainder of the column (carrying vitamins D and sterols) is replaced in its suction flask and eluted with 25 ml. of ether.

"The combined filtrate and eluate are evaporated to dryness under reduced pressure and taken up in 10 ml. of purified chloroform. To 1 ml. of this solution are added 10 ml. of the $SbCl_3$ reagent. The flask is swirled for 30 sec., the absorption cell filled, and the extinction determined on the Bausch & Lomb visual spectrophotometer exactly 3 min. after starting to add the reagent to the vitamin sample. The sample at this point contains vitamins D and sterols.

"To correct for the absorption at 500 mμ due to the sterols present, another 1 ml. aliquot of the chloroform solution of vitamins D plus sterols is evaporated to dryness and taken up in 5 ml. of 1 to 2 Skellysolve-benzene mixture. A tightly packed 1.5 cm. Superfiltrol column is wetted with 5 ml. of the mixed solvent and the sample added, followed by the addition of 5 ml. of solvent to rinse the flask and then 50 ml. to elute the sterols. A lavender-blue band carrying the vitamins D is fixed in the upper portion of the adsorption column. The sterols pass into the filtrate. The filtrate is evaporated to dryness under reduced pressure and the residue taken up in 1 ml. of chloroform. To this are added 10 ml. of the $SbCl_3$ reagent, using a 2 cm. absorption cell. The extinction is determined at 500 mμ exactly 3 min. after mixing.

"From these two extinction values, the $E^{1\%}_{1\,cm}$ is calculated for vitamins D and sterols combined and for sterols alone. The difference between these two values gives the $E^{1\%}_{1\,cm.}$ for the vitamins D in the original sample. This value, multiplied by the factor 19,300 . . . gives the potency in U.S.P. units/g. of oil."

The agreement between this colorimetric and the usual biological method was found to be good for oils ranging from 5000 units/g. and up in potency. For weaker oils the method is not so satisfactory.

III. Vitamin E

The chemical methods, with all their various modifications, suggested for the determination of tocopherols are based on the oxido-reductive quality of tocopherols and utilize 3 different color reactions for practical colorimetric analysis.

(a). The method originally proposed by Furter and Meyer (109) is based on the oxidation of tocopherols to the red orthoquinone with concentrated HNO_3.

(b). The method originally proposed by Emmerie and Engel (110) is based on the reducing property of tocopherol toward $FeCl_3$. The ferrous salt which is formed in the reduction is determined colorimetrically with *a, a'*-bipyridine.

(c). The method originally proposed by Scudi and Buhs (111) is based on the decolorization of 2, 6-dichlorophenol indophenol with tocopherol.

As in most chemical methods of vitamin analysis, all these colorimetric methods suffer to a various degree from a lack of specificity because, in general, the reactions and color changes upon which they depend are subject to interference by other substances present, some of them unidentified.

Attempts were made and modifications put forward to adapt several of the chemical methods to the desired use.

1. METHOD OF FURTER AND MEYER (109), MODIFIED BY CHIPAULT, LUNDBERG AND BURR (112), WITH SPECIAL REFERENCE TO THE DETERMINATION OF TOCOPHEROLS IN FATS AND OILS

Requirements.

(a). Spectrophotometer or photoelectric colorimeter (the latter to be used only when large amounts of tocopherol are present).

(b). Glass apparatuses of special design (see Figs. 4 and 5).

Reagents.

Air-free KOH solution. Air-free methanol. Ethanol (95% and absolute). Absolute *n*-butyl alcohol. U.S.P. ether (peroxide free). Benzene. Petroleum ether (low boiling). Glacial acetic acid. Conc. HNO_3. 33.2% H_2SO_4 (sp. gr. 1.249 at 15.5° C.). Methylene blue. Anhydrous Na_2SO_4. Brockmann alumina (Merck). Florisil (minus 200 mesh, Floridin Co., Warren, Pa.). Granulated zinc. Air free water. N_2.

According to Chipault, Lundberg and Burr (112), neither the method of Emmerie and Engel (110) and its modifications by Parker and McFarlane (113), by Devlin and Mattill (114) and by Hines and

Mattill (115); nor the method of Scudi and Buhs (111) is applicable to fats since errors are caused by sterols and other interfering substances in fats. They were, however, able to increase considerably the sensitivity of the method of Furter and Meyer (109) by eliminating the interference caused by substances present in fats and different from tocopherols. The first part of the modification proposed by Chipault, Lundberg and Burr is based on the suggestion of Binnington and Andrews (116) and consists in the chromatographic separation of the *o*-quinones. As a further important step, Chipault, Lundberg and Burr added the spectrophotometric (colorimetric) determination of the purified *o*-quinones with the help of a solution of leuco methylene blue. With this technic it is possible to measure 20 γ of tocopherol with an estimated average deviation from the mean of less than 10%. The method does not distinguish α-, β-, γ-, and δ-tocopherols. It is necessary to resort to animal assay coupled with the results of chemical analysis to ascertain whether a single tocopherol or a mixture is present.

The analytical procedure may be conveniently divided into 3 steps: (a) saponification of the fat and the preparation of the unsaponifiable fraction, (b) conversion of the tocopherols in the unsaponifiable fraction to purified *o*-quinones, and (c) estimation of the tocopherols by reaction of the *o*-quinones with leuco methylene blue.

(a). Saponification and Preparation of Unsaponifiable Fraction. Since the tocopherols are sensitive to oxidation in the presence of alkalies, saponification must be conducted with air-free alkali in an inert atmosphere that must be maintained throughout the subsequent extraction of the unsaponifiable matter.

Air-free alkali is prepared by dissolving 400 g. of KOH pellets in 2 l. of absolute methanol in an Erlenmeyer flask, using a stream of N_2 to obtain the necessary agitation. The solution is filtered under suction through a sintered glass funnel to remove insoluble carbonates, and then transferred under N_2 into the storage bottle illustrated in Fig. 4c. A gentle stream of N_2 is passed through the delivery tube in a reverse direction for 1 hr. to insure complete removal of oxygen. N_2 pressure for delivery of the reagent is applied by means of a rubber balloon, previously filled with N_2. This balloon may be refilled when necessary through the side stopcock.

The safest procedure is to purify the N_2 used in this and all subsequent operations by passage through Fieser's reagent[1] (117) or over a heated copper gauze. It is highly important that all possible precautions

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[1] Composition of Fieser's solution: sodium hyposulfite 16, NaOH (stick) 6.6, anthraquinone-β-sulfonate (95%) 2, dissolved in 100 ml. of water.

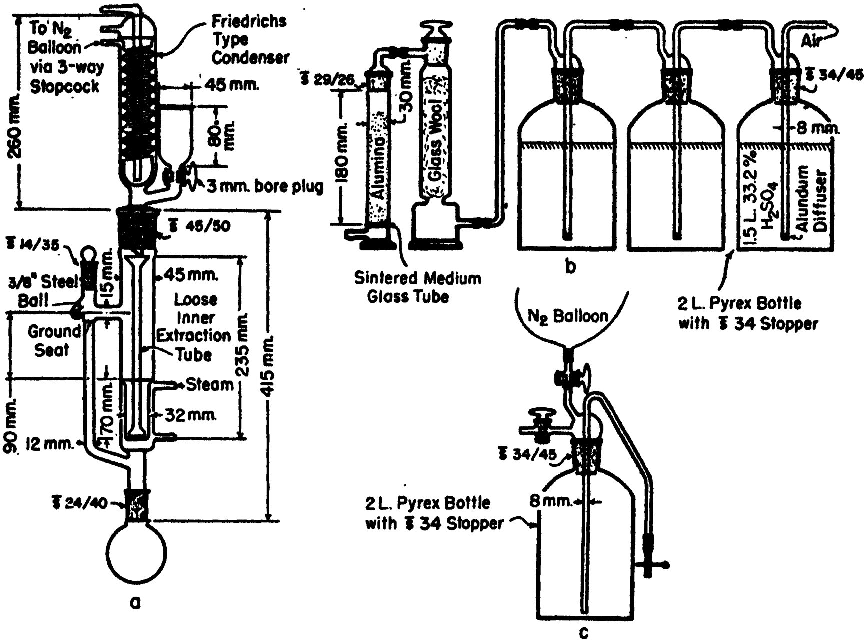

Fig. 4. Apparatus designed by D. S. Binnington for use in determination of tocopherols (after Chipault *et al.* (112)).

be taken to completely prohibit contact of the tocopherols with oxygen, particularly when alkali is present.

Saponification and extraction of the unsaponifiable matter is carried out in the apparatus shown in Fig. 4a. The condenser is removed and between 8 and 9 g. of melted fat are transferred directly into the apparatus through the top. With the condenser back in place, the system is evacuated with a good water pump. N_2 is admitted from a storage balloon and the evacuation is repeated. Seventeen ml. of the air-free KOH solution and 13 ml. of air-free CH_3OH are added through the top funnel with the aid of suction and the process of alternate evacuation and flushing with N_2 is repeated 5 times. (With liquid fats, as much as 10.5 g. of the fat and a corresponding increase in the amount of alkali may be used, and the addition of the excess pure methyl alcohol is unnecessary.)

With the apparatus under a slight positive N_2 pressure, maintained by the balloon, the steel ball valve is transferred from the side pocket to its seat with a powerful hand magnet or an electromagnet. Steam is then passed through the jacket. After 45 min., and with the steam still circulated through the jacket, 50 ml. of air-free water (stored under N_2) are added slowly through the top funnel, the apparatus being carefully vented to the air *via* the three-way stopcock. The steam is turned off and an additional 20 ml. of air-free water are rapidly added. The apparatus is returned to the nitrogen pressure of the balloon and the steel ball valve returned to the side pocket.

After the apparatus and contents have cooled to room temperature, and not before, peroxide-free U.S.P. ethyl ether is added until the lower flask is one-third filled. The ether is permitted to boil on a steam bath for 3.5 hr. This time is ample for complete extraction if the temperature of the condenser water does not exceed 15° C. Any tendency toward lumping caused by the formation of a 2-phase system in the lower flask is best minimized by introducing a few ml. of absolute alcohol into the flask before the saponification is begun. A pinch of washed sand introduced at the same time promotes a smoother boiling of the ether.

The extract is transferred to a 250 ml. Squibb separatory funnel with a minimum of exposure to the air. It is immediately washed, gently at first, with air-free water until free from alkali as shown by testing the wash water with phenolphthalein. Finally, the extract is dried over anhydrous Na_2SO_4 and filtered. The Na_2SO_4 is washed several times with small portions of ether and the washings are added to the filtrate.

(b). Conversion of the Tocopherols to o-quinones. The extract is evaporated to dryness, the residue being finally contained in a small transfer flask, (Fig. 5c). Two ml. of benzene are added and the sterols and carotenoids are largely removed by adsorption on purified Florisil, using the apparatus in Fig. 5b. The Florisil is purified and used essentially according to the procedure[2] described by Emmerie and Engel (118), except that following purification it is dried in an oven at 120° C. for 3 hr. The dimensions of the column used in this step are 5 × 100 mm. Following passage of the solution, the transfer flask and column are washed with three 2 ml. portions of benzene. The percolate is run directly into a 1 × 4 in. test tube provided with a lip for pouring. The benzene is removed on a water bath under a stream of N_2. Ten ml. of absolute *n*-butyl alcohol are added to dissolve the residue, warming, if necessary, to insure complete solution. When cool, 2 ml. of conc. HNO_3 are added from a pipette into the center of the solution to prevent local concentration and are thoroughly mixed. The tube is immediately placed in a vigorously boiling water bath for 2 min. (± 5 sec.) and then quickly placed in a bath of ice water and cooled to room temperature as rapidly as possible. The time of heating is critical and the 2 min.

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[2] Florisil is heated for 1 hr. with conc. HCl over boiling water. After decantation the earth is left standing for some hours with fresh conc. HCl at room temp., with occasional agitation. This HCl wash is repeated 3-4 times. Afterwards the earth is washed with water, ethanol, and benzene.

period specified is optimum for the apparatus and conditions described.

Without delay, the reaction products are transferred with the aid of three 5 ml. portions of absolute ethanol to a 250 ml. separatory funnel containing 150 ml. of H_2O. Twenty-five ml. of low boiling petroleum ether are added, and the contents of the funnel are shaken well and permitted to stand for 1 hr. The lower layer is drawn off and discarded. The petroleum ether layer is washed twice with 100 ml. portions of H_2O using gentle agitation, and then dried by filtration through a small amount of anhydrous Na_2SO_4, into a smaller transfer flask. The funnel and Na_2SO_4 are rinsed with an additional small quantity of petroleum ether. The filtrate is evaporated to dryness on a tepid water bath (35° C.) in a stream of N_2. When the petroleum ether has disappeared, the stream of N_2 is continued for 3-5 min. but no longer than is necessary to remove the odor of the last traces of butyl nitrate.

The residue is taken up in 2 ml. of low boiling petroleum ether and drawn through a column of Merck's Brockman alumina (American variety) containing 12% moisture. The apparatus shown in Fig. 5b is again used, this time with a column with dimensions of 3×75 mm. The transfer flask and column are rinsed with another 2 ml. of petroleum ether. The red o-quinone is strongly adsorbed near the top of the column immediately below a layer of impurities. The column is then carefully washed with small amounts of benzene until the red zone has almost reached the bottom of the column. When the original sample contains as little as 20 γ of tocopherol, the red zone is barely visible and care must be taken not to overlook it. The last of the benzene is drawn out and the column is washed once with petroleum ether, and dried by suction to such an extent that the top of the column containing the strongly adsorbed impurities may be poured off. The remainder of the column containing the red zone is eluted with a small amount of peroxide-free ethyl ether directly into the side pocket of the spectrophotometer tube shown in Fig. 5a. If it is judged that too much quinone is present for the methylene blue determination, the eluant is received in a 10 ml. volumetric flask instead and an aliquot taken for the final step.

The moisture content of the alumina is important and various types of alumina have widely different optimum moisture levels. With the American variety of Brockman alumina (made by Merck) a moisture content appreciably less than 12% results in a too strong adsorption of the quinones and more than 12% prevents adequate separation of impurities. The proper moisture content may be attained by exposing the dried alumina to the atmosphere above 33.2% sulfuric acid (sp. gr. 1.249 at 15.5° C.) either in thin layers in a desiccator or in a tower in the train shown in Fig. 4b. In either case, the moisture content of the alumina should be determined by measuring the loss in weight on ignition in a muffle for 1 hr. at 450-550° C.

When relatively large amounts of tocopherol are present, as in some vegetable oils, it is possible to conclude the analysis with a colorimetric measurement of an alcohol solution of the *o*-quinones, using an Evelyn colorimeter equipped with a 490 mμ filter. In such cases, larger adsorption columns and a two-stage adsorption may be desirable since vegetable oils usually give rise to more pigments than animal fats.

(c). Reaction of Quinones with Leuco Methylene Blue. Methylene blue is dissolved in 95% alcohol to produce a few ml. of concentrated solution. After filtering, the solution is diluted with a mixture of 80 parts by volume ethyl alcohol (95%), 15 parts water, and 5 parts glacial acetic acid, to produce a stock solution of methylene blue having an optical density of approximately 2.0-2.5/cm. of cell thickness at 6500 Å. Attainment of the proper concentration is achieved with the aid of spectrophotometric measurements on aliquots at still greater dilution against a blank of solvent mixture.

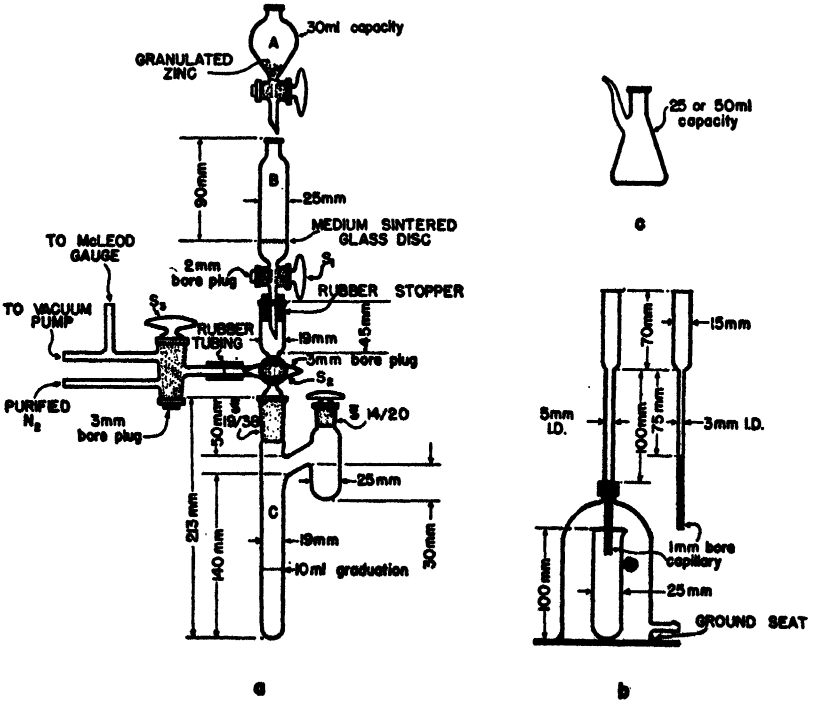

Fig. 5. Accessory apparatus used in methylene blue method (after Chipault *et al.* (112)).

Fig. 5 depicts the apparatus used in the next phase of the analysis. Fifteen ml. of the stock methylene blue solution are introduced into the separatory funnel A containing a few grams of granulated zinc. The reaction mixture is protected from oxygen by passing a gentle stream of N_2 up through the stem of the funnel.

While the preliminary reduction of the methylene blue is thus going on, the ether eluant containing the *o*-quinones is introduced into the side pocket of the spectrophotometer tube C. The solvent is evaporated at room temperature by means of a gentle stream of N_2. The application of a stream of N_2 and the subsequent application of vacuum to the quinones should be continued only as long as is necessary; prolonged vaporizing conditions cause low results.

In chamber B, containing a sealed-in, sintered glass plate, a pinch of zinc dust is placed, and the chamber is closed by means of a rubber stopper. Stopcock S_1, the three-way T-bore stopcock S_2, and the three-way two-angle-bore stopcock S_3 are opened so that the entire apparatus is evacuated by a mechanical oil pump. When the McLeod gauge in the vacuum line shows that the pressure has dropped to 10 or 20 μ, chamber C is closed off and N_2 is admitted to chamber B. The stopper is removed from B, and a flow of N_2 into the chamber through the system of stopcocks is continued while the almost reduced methylene blue is admitted through the top from A. After thoroughly mixing the contents of B, the stopper is replaced, S_1 is closed, and the small chamber above S_2 is evacuated. When the pressure has fallen to 20 μ or less, S_2 is turned to close off the line to S_3, and the rubber connection between S_2 and S_3 is disconnected. S_1 is opened and 10 ml. of reduced methylene blue is permitted to flow into C. S_2 is turned to close off C, the apparatus is removed from its support, and B is disconnected. With the side pocket in the uppermost position to avoid contact of the methylene blue with the quinones, C is tipped back and forth gently 30 or 40 times or until a constant reading is obtained in the spectrophotometer. Usually the leuco methylene blue has a transmission of more than 90% at 6500 Å when compared to 95% alcohol in square cuvettes of 1.25 cm. thickness.

The leuco methylene blue is then allowed to mix with the quinones, shaken vigorously, and a spectrophotometer reading made. Alternate shakings and readings are continued until a minimum transmission at 6500 Å is obtained. Thereafter, a fading of the blue color begins, but is so slow that no correction is required. The optical density corresponding to the minimum transmission is referred to an empirical reference curve to obtain the tocopherol content of the sample.

Preparation of Reference Curve. Since there are small losses of one kind or another during the analytical process, it is necessary to prepare an empirical reference curve relating the measured optical densities to the original tocopherol contents. The data for this curve must be obtained by performing the complete analysis on samples of fat containing known amounts of tocopherol. This purpose is best served by using as basic substrate lard from which tocopherols have been removed and to which graded amounts of pure α-tocopherol were added. The analysis is carried out on the fat as negative control and on the samples containing the various amounts of tocopherol as supplements. The tocopherols are removed by dissolving 200 g. of lard in 1000 ml. of low boiling petroleum ether and passing through two columns of activated alumina 3 × 34 cm. (chromatographic alumina, Grade A, minus 80 mesh, Aluminum Ore Co., and Merck's Brockman Alumina). The solvent is removed under vacuum. Inasmuch as this residue still contains small amounts of tocopherol, the optical densities obtained upon analysis of such treated lard (as negative control) should be applied as corrections to the optical densities found for the samples containing added tocopherol. The corrected optical densities, D_c, used for the calibration data, are calculated according to the following formula:

$$D_c = D_s - \frac{W_s}{W_b} D_b, \text{ where}$$

D_s = measured optical density for the sample
D_b = measured optical density for the control (substrate)
W_s = weight of sample
W_b = weight of control

The corrected optical densities and the added amounts of tocopherol are related within experimental error by a straight line, which may be used as the reference curve for the calculation of results.

It is claimed (112) that the rather critical and detailed character of the procedure is more than offset by the high degree of specificity and sensitivity obtained.

2. THE METHOD OF EMMERIE AND ENGEL AND ITS MODIFICATIONS

The iron-α, α′-bipyridine reaction, as first introduced by Emmerie and Engel (110), is more sensitive than the method of Furter and Meyer (109), but is limited in its use because of the marked depressing effect of fats on the iron-bipyridine color and of the interference by substances such as cholesterol, vitamin A and carotenoids with the color reaction (110-115). To overcome these difficulties, elaborate procedures were used to separate the tocopherol from the fat and other interfering substances. Emmerie and Engel (110, 118) recommended that oils be saponified, and that the carotenoid pigments be removed from the unsaponifiable fraction by adsorption on Floridin XS earth before the test is carried out.

Because of the known instability of tocopherols in alkaline solutions, the saponification procedure might easily entail losses (113, 114), preventable only by intricate precautionary measures such as described above for the method of Chipault, Lundberg and Burr (112). To avoid this difficulty, Parker and McFarlane (113) proposed to work with unsaponified oils and recommended the removal of interfering substances (vitamin A, carotene and cholesterol) through treatment of a dilute petrol ether solution of the oil to be analyzed with 85% H_2SO_4 and consecutive washing of the petrol ether-phase with dilute KOH solution (1%). However, this acid-alkaline treatment does not accomplish the complete removal of sterols or reacting degradation products of cholesterol (114) and other interfering substances. Thus, it is not astonishing that the method as recommended by Parker and McFarlane (113) has not given—at least in the hands of other research workers (112, 114, 119)—consistent and reproducible results.

More recently, the original Emmerie-Engel method has undergone several modifications with special reference to its use for oils and fats. One of these modifications used the "increment" technique (119), the other increased the sensitivity of the method with the use of pyrogallol and activated alumina (120).

The recognition of δ-tocopherol (121) as one of the commonly occurring tocopherols necessitated a further modification (122) of the Emmerie-Engel method.

For substances such as extracts from muscle, liver or fecal material, in which neutral fat is not the predominant constituent as it is in fats and oils, Mattill and his associates (114, 115) recommended a modification of Emmerie-Engel adsorption method (118) combined with the Parker and McFarlane acid-alkali treatment (113). It is claimed (123) that the "increment" technique (119) is also applicable to this class of substances. Furthermore, it is simpler than the method used by Mattill and his associates (114, 115), and in better accord with the results of bioassays.

Pure tocopherol may be analyzed with the Emmerie-Engel method in its original form.

A. DETERMINATION OF PURE TOCOPHEROL BY THE COLORIMETRIC METHOD OF EMMERIE AND ENGEL (110)

Reagents.

Absolute ethanol, purified as follows (113). To 100 ml. of alcohol add about 2 g. of ferric nitrate. Let stand in the dark for at least 48 hr.

Distill in subdued light and store the alcohol in a brown glass bottle.

Solution of ferric chloride (ferrum sesquichloratum cryst. Merck) 0.2% in pure ethanol (solution must be freshly prepared).

Solution of α, α′-bipyridine (Merck) 0.5% in pure ethanol.

The solutions of ferric chloride and α, α′-bipyridine may be replaced by a mixed solution of 250 mg. of $FeCl_3$ and 500 mg. of α, α′-bipyridine in a liter of glacial acetic acid (114). With glacial acetic acid as solvent (instead of ethanol) the reagent becomes insensitive to light and remains stable for a long time (114).

Method. To 1 ml. (or more) of solution (containing 0.1-0.4 mg. of α-tocopherol) are added 1 ml. of $FeCl_3$ solution and 1 ml. of bipyridine solution (or 1 ml. of the mixed solution in glacial acetic acid). The volume is made up to 25 ml. and the red color of the ferrous-bipyridine complex (with the absorption maximum at about 515 mμ) is measured after 10-15 min. in a photoelectric colorimeter with the use of an appropriate filter. A control with $FeCl_3$ and bipyridine is also prepared and serves as blank in the usual manner. The result is obtained from the calibration curve prepared with the use of graded amounts of pure synthetic α-tocopherol. The maximum intensity of color is reached almost immediately on adding the reagents, and no appreciable increase relative to the blank is noted after the solution is allowed to stand in the dark for 15 min.

The rate of color formation for pure tocopherols in acetic acid is not uniform. With γ-tocopherol complete oxidation is obtained only in about 40 min. β-Tocopherol is oxidized by $FeCl_3$ in acetic acid at a rate intermediate between that of α- and γ-tocopherols. δ-Tocopherol requires a still longer period than γ-tocopherol (122).

B. THE "INCREMENT-METHOD" FOR THE DETERMINATION OF TOCOPHEROLS WITH IRON-BIPYRIDINE REAGENT IN FATS (119) AND IN TISSUE EXTRACTS (123)

Reagents.

Skellysolve B (SSB), purified by twice shaking with concentrated H_2SO_4, washing with water, 10% Na_2CO_3, four times with water, drying over anhydrous Na_2SO_4 and distilling in an all-glass apparatus.

Acetone (reagent grade).

Sesame oil. A sample kept in laboratory for at least several weeks is used. 0.1 ml. of the oil made to 2 ml. with Skellysolve should have a $-\log T$ value of less than 0.002 with 10 ml. of the iron-bipyridine reagent. If the $-\log T$ is higher, correction must be made.

Iron-bipyridine reagent. Solution of 250 mg. of $FeCl_3.6H_2O$ (reagent grade) and 500 mg. of α, α'-bipyridine in 1000 ml. of glacial acetic acid ((114), see also above).

Synthetic *dl*-α-tocopherol.

Apparatus: Coleman Universal Spectrophotometer.

Method.

(1) For Fats and Oils

The tests are generally carried out in the following way. SSB solutions containing 10% of the respective fats and oils and a second set of petroleum ether solutions containing from 25 to 200 γ of α-tocopherol/ml. are prepared. Two ml. of SSB are pipetted into the comparison cell of the spectrophotometer, followed by 10 ml. of iron-bipyridine reagent. Into an identical cell are pipetted 1 ml. of the oil solution and 1 ml. of SSB containing from 0 to 200 γ of α-tocopherol, again followed by 10 ml. of reagent. From the instant when the reagent is added, the development of the color is recorded approximately at minute intervals within an accuracy of 3 sec. in the timing and of 1% or less in the extinction for readings of 0.1-0.5 when a 1.0 ml. square cell is used. Maximum readings within the first 10 min. after addition of the reagent are plotted.

The special technique recommended for this modification of the original Emmerie and Engel method is based essentially on the observation (119) that fats have a distinct depressing effect on the color reaction. The standard curve given by amounts of from 0 to 200 γ of pure α-tocopherol follows the Beer-Lambert law up to an extinction of approximately 0.4 which corresponds to about 100 γ of tocopherol. By adding equal amounts, for instance 0.1 ml., of various fats to the same series of tocopherol solutions, the curve still remains linear but the slope becomes smaller, and is characteristically different for the various fats used. This means that, for example, in the presence of 0.1 ml. of sesame oil, it requires 200 γ of tocopherol to produce the extinction given by 33 γ in a pure α-tocopherol solution. The fact that only the nature and the concentration of the fats and oils, and not the tocopherol: fat ratio, influences the results in the range selected for the determination, is clearly indicated by the further observation that, in contrast to the linear curves obtained with samples containing varying concentrations of tocopherol and constant amounts of fats, the curves are far from being linear when the conditions are reversed and constant amounts of tocopherol and varying amounts of fats are used.

In the light of these observations, *calculation* of the tocopherol content in oils and fats containing negligible amounts of equally color-giving vitamin A and carotenoids may be carried out with the usual extrapolation method as illustrated by the following example. The linear curve representing the effect of tocopherol supplements of 0-200 γ on the extinction of the iron-bipyridine color in the presence of constant amounts of the test-sample (fat or oil) is drawn (Fig. 6). From the original reading and the slope of the curve, it is possible to calculate the original concentration of tocopherol by means of the similar triangles ABO and ACF with the extinction E_1 (OB) for the unknown (X) and E_2 (CF) for $X + a$ γ of α-tocopherol. X as the only unknown can be calculated from the following equation:

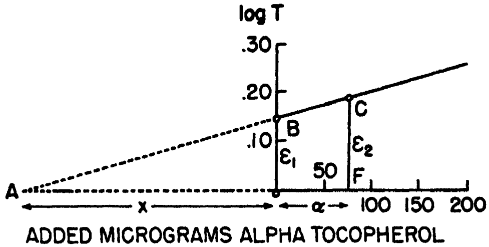

Fig. 6. Sesame oil with added tocopherol graphically presenting the extrapolation method (after Kaunitz and Beaver (119)).

$$E_1 : X = E_2 : (X + a)$$

For fats and oils in which the presence of vitamin A and carotenoids complicates the determination of tocopherols, the following correction may be used (119). First the amount of carotenes and vitamin A is determined by standard methods (p. 158 ff.) and the iron-bipyridine method is carried out as above with and without addition of known amounts of carotene and vitamin A. Thus, the extinction given by the amount of carotenes and vitamin A originally present can be measured and subtracted from the total extinctions; thereupon, the calculation of tocopherol proceeds as above. In general, the error caused by carotenes does not exceed 10% of the tocopherol values even if no correction for the carotenes is introduced.

Among other substances possibly interfering with the method, sterols have to be taken into consideration. However, addition of 100 mg. of recrystallized cholesterol to the tocopherol-fat mixtures has been found (119) without pronounced effect on the extinctions obtained with the iron-bipyridine reagent.

(2). In Tissue Extracts

Example: muscle (123)

Thirty to 50 g. of muscle, weighed to 0.2 g., are emulsified with about 100 ml. of acetone in a Waring blendor for 6 min. and washed into a centrifuge tube with an additional 350 ml. of acetone. Determinations can be made with 5 g. of muscle but with a larger error. After 24 hr. the tube is centrifuged, the residue once more extracted with acetone, once with one-half acetone and one-half Skellysolve, and once with one-third acetone and two-thirds Skellysolve. The combined extracts are transferred to a 3 l. separatory funnel, and water is added until 2 distinct layers are formed. The Skellysolve phase is saved, the aqueous phase is twice extracted with 200 ml. of Skellysolve, and the combined Skellysolve extracts are washed 3 times with 500 ml. of distilled water, centrifuged, and evaporated in an all-glass apparatus under reduced pressure in an atmosphere of nitrogen. The residue is washed into a 25 ml. flask with approximately 20 ml. of Skellysolve; 1-2 ml. of sesame oil (according to its color-depressing properties) is added and the volume made to 25 ml. with Skellysolve. The mixture should be slightly yellow and entirely clear. In rare instances a brown, strongly reducing pigment is noted which interferes with the determination. The pigment can often be removed by washing the Skellysolve extract with a 2% Na_2CO_3 solution.

For the carotene determination, the $-\log T$ value of the optically clear solution is determined in a Coleman Universal spectrophotometer or in a Beckman spectrophotometer with Skellysolve as blank at 4400 Å. The concentration is read from a calibration curve.

Two standard solutions containing 40-50 and 80-100 γ of synthetic *dl*-α-tocopherols/ml. of Skellysolve (determined to 1 γ) are prepared.

Aliquots of the muscle extract of 1 ml. each are pipetted into 3 test tubes; to one of these is added 1 ml. of Skellysolve, and to each of the other two 1 ml. of the standard tocopherol solutions. To each test tube are added 10 ml. of iron-bipyridine reagent; 2 ml. of Skellysolve containing the same amount of the same sesame oil as the unknown plus 10 ml. of iron-bipyridine reagent serve as a blank. The test tubes are kept in the dark. Approximately every minute a $-\log T$ reading is taken, starting with the sample to which the higher tocopherol concentration was added. As soon as the maximum reading is reached, the second tocopherol-containing sample is examined, and finally the sample to which no tocopherol had been added. This procedure is possible because it was found experimentally that the maximum reading appears fastest in the sample with the highest tocopherol concentration. The dilutions of the extracts should be such that none of the — log T readings exceeds 0.4.

The uncorrected value, X, for the number of micrograms of tocopherol/ml. of diluted extract is given by the expression

$$X = \frac{(e_1 n)}{e_2 - e_1}$$

where e_1 represents the $-\log T$ value of the unknown, e_2 that of the unknown plus tocopherol, and n the number of micrograms of tocopherol added. For each γ of carotene/ml. of diluted extract, 4.8 is subtracted from the value for X. All results are finally computed on the basis of mg. of tocopherol/1000 g. of muscles.

A possible error of approximately 2-5% is caused by the presence of the normal amounts of vitamin A in muscle extracts. This estimate is based upon the experimental fact that the reductive power of 1 I.U. of vitamin A was found to be equivalent to 0.24 ± 0.02 γ of α-tocopherol and that 1000 g. of muscle contain roughly 4000 I.U. of vitamin A. The higher vitamin A content in liver extract may require special correction.

a. Modification of the Emmerie-Engel Method with the Use of Pyrogallol and Activated Alumina (120). The unavoidable losses of vitamin E which occur in oils and fats during the customary procedure of saponification may be avoided when the saponification is carried out in the presence of pyrogallol. The resulting unsaponifiable fraction contains, in addition to tocopherols and carotenoid pigments, varying amounts of other irrelevant substances which are capable of reducing $FeCl_3$. They can be removed by adsorption on a column of activated alumina, under conditions which are carefully adjusted to allow free passage of α-tocopherol. It appears that at least one of the tocopherol-isomers, β-tocopherol, is partially adsorbed on the column. In practice, however, this inefficient passage through the column tends to compensate for its much lower biological activity.

Carotenoid pigments which are not adsorbed on the column may be present in sufficient quantity to necessitate a correction in the total $FeCl_3$-reducing substance. First the amount of carotenoid pigments in the unsaponifiable fraction must be determined. Inasmuch as 1 γ of carotene was found to have reducing power equal to 2.5 γ of α-tocopherol, the total amount of carotene found is multiplied by 2.5 and the figure obtained deducted from the uncorrected vitamin E value as determined in the unsaponifiable fraction.

Reagents.

Pyrogallol solution 5% (w./v.) freshly prepared with ethanol as solvent.

KOH solution (for saponification). Aqueous solution containing 4 g. of KOH + 2.7 ml. H_2O.

Solution of 250 mg. of $FeCl_3.6H_2O$ and 500 mg. of α, α′-bipyridine in 1000 cc. of glacial acetic acid (106, see also above).

Ethanol

Ethyl ether

Light petroleum (b.p. 40-60° C.).

Aluminum oxide (British Drug Houses Ltd. for chromatographic analysis). Activated by heating at 100° C. in an air oven for 2-3 hr. before use.

It is essential to standardize each new batch of alumina against synthetic α-tocopherol or various oils. Brockmann alumina was found to adsorb too strongly and failed to allow the quantitative passage of α-tocopherol.

Light petroleum + ethanol solvent. For chromatography, 1% (w./v.) of absolute ethanol in petroleum ether.

Method.

Saponification. One g. of oil is weighed in a conical flask, 2 ml. of 5% pyrogallol are added, and the mixture is heated to 86° C. on a water bath. After the addition of 0.44 ml. of the KOH solution, the flask is shaken in the bath for about 2 min., which usually causes complete saponification. To this mixture 8 ml. distilled water are added, the unsaponifiable matter is extracted in a separating funnel by shaking vigorously first with 10 ml. of ether and then with 15 ml. of ether. The 2 ether extracts are combined and washed twice, each time with 5 ml. of 0.5% aqueous KOH, vigorous shaking being avoided at this stage in view of the danger of emulsions. The ether extract is next washed several times with 10 ml. portions of distilled water, with only gentle shaking for the first and second times. Adequate washing of the extract is indicated by the quick separation of layers after vigorous shaking. The ether extract is evaporated on a water bath under reduced pressure, and the unsaponifiable matter dissolved in a volume of ethanol, so that 1 or 2 ml. of such solution are equivalent to about 1 mg. of $FeCl_3$-reducing substances. The solution at this stage may be kept overnight in a refrigerator without harm.

If duplicate estimations are to be made, unsaponifiable matter equivalent to 2400-4000 γ of reducing substances should be prepared. It is usually necessary to take more than 1 g. of oil, to increase proportionately the amounts of all the reagents used, and sometimes to extend slightly the time of saponification.

Chromatographic Analysis. A portion of the solution of unsaponifiable matter, containing about 1 mg. of the reducing substances, is evaporated under reduced pressure to dryness on a hot water bath, and care is taken to remove all ethanol. (*a*). *Solvent.* Thirty ml. of light petroleum + 1% ethanol solvent are prepared carefully for each chromatographic separation. (*b*). *Preparation of Al_2O_3 column.* Activated Al_2O_3 is packed in a tube of 10 mm. diameter and about 120 mm. long, so that the length of the column is 50 mm. and diameter 10 mm. Ten ml. of light petroleum ether + 1% ethanol solvent are passed through the column, which is now ready for use. For each estimation a new column should be made.

A portion of the unsaponifiable matter is dissolved in 5 ml. of the solvent mixture and passed through the column, which is then washed with 15 ml. of the solvent. Throughout the analysis the top of the column should not be allowed to become dry, and the rate of filtration should be 70-90 drops/min. The "unadsorbed fraction" of 20 ml. volume is collected in a clean flask, the solvent evaporated under reduced pressure on a hot water bath, and the residue dissolved in ethanol and made to a volume of not more than 25 ml. This solution is now ready for colorimetric estimations.

Colorimetric Estimations. (*a*). *Estimation of Carotenoid Pigments.* In the unadsorbed fraction, mainly carotene is found and is estimated either directly by its natural yellow color or by one of the usual methods (page 177). The quantity of carotene is expressed in γ/1 g. of oil. (*b*). *Estimation of Vitamin E.* A portion of the solution, containing preferably between 100 and 160 γ of $FeCl_3$-reducing substances is used for analysis which is carried out according to the original method of Emmerie-Engel (see above page 195). For correction of carotene present the amount of carotene (per g.) is multiplied by the conversion factor 2.5 and deducted from the total figure (expressed per g. test-substance).

b. Modification of the Emmerie-Engel Method in the Presence of δ-Tocopherol (122). δ-Tocopherol is present in large amount (approx. 30%) in soybean oil; it is found also (5%) in wheat germ oil and there is evidence of its occurrence in cottonseed and peanut oils (121). In contrast to α-, β- and γ-tocopherol this newly discovered tocopherol is oxidized only very slowly by the iron-bipyridine reagent. The oxidation is especially slow when the acetic acid reagent ($FeCl_3$ and bipyridine dissolved in glacial acetic acid, (114). See also above) is applied. Using the ethanol-solution of iron-bipyridine the differences in rate of oxidation among the pure tocopherols are less pronounced. Color formation for α-, β- and γ-tocopherols is substantially complete with the ethanol-reagent in 2 min., with only a small increase occurring when the reaction time is extended to 10 min. On the other hand, in the case of δ-tocopherol this delayed increase may reach 30% of the color intensity found at 2 min.

In the light of these findings it appears to be advisable to substitute the ethanol-reagent[8] for the acetic acid reagent in the iron-bipyridine method of Emmerie and Engel or in its various modifications previously described. Stern and Baxter recommend the following procedure (122):

A 1 ml. aliquot of the sample containing 50-150 γ of tocopherol in purified ethanol (for concentrates) or petroleum ether (Skellysolve B for low-potency preparations containing glycerides) is added to a 2 oz. glass-stoppered bottle painted black, followed in order by 1 ml. of bipyridine reagent, 1 ml. of $FeCl_3$ reagent (both made up in purified absolute ethanol), and 22 ml. of purified ethanol. The last is added from a 50 ml. buret. When 17 ml. of ethanol have been added, a timer equipped with a second hand is started. This operation can be completed in the time necessary for the remainder of the 22 ml. to run into the reaction bottle. The stopper is inserted, the solution is swirled gently, and the bottle is set aside for 2.5 min. A blank previously prepared (1 ml. of ethanol, 1 ml. of each reagent, then 22 ml. of ethanol) is used to adjust the galvanometer of the Evelyn colorimeter to read 100, using a No. 520 filter. Approximately 10 sec. before the completion of the 2.5 min. period, the test solution is poured into an Evelyn tube in the colorimeter; the tube is covered with a black cap; the galvanometer reading, G, is determined at 2.5 min.; and $L = (2 - \log G)$ is calculated. The tocopherol content is determined with a calibration curve (relating L to the tocopherol concentration) which is prepared with pure natural α-tocopherol or with a 50:50 mixture of natural α- and β-tocopherols. With galvanometer readings of 25 to 70, L is a linear function of the tocopherol concentration. Lower galvanometer readings should be avoided since they indicate an insufficient excess of $FeCl_3$.

Excessive exposure to light of either the blank or the test sample must be avoided because this causes the formation of spurious color. Painting the reagent bottles black, putting a black cap over the tube while it is in the galvanometer, and the use of a low-wattage bulb in the galvanometer will prevent this in part.

Interference by neutral fat and various pigments may be minimized by the increment method of Kaunitz and Beaver (119), by chemical purification (113, 115, 118), or by molecular distillation of the tocopherols from fats and oils of low tocopherol content (123a).

[8] For composition see page 196.

C. DETERMINATION OF THE TOCOPHEROLS (AND THE TOCOPHERYLQUINONES) BY A COLORIMETRIC OXIDATION-REDUCTION METHOD (111)

Under a variety of conditions tocopherols are readily converted to the corresponding tocopherylquinones which are devoid of vitamin E activity (124). Scudi and Buhs have previously described a colorimetric oxidation-reduction method for the determination of vitamin K (125, 126). This method measures not only vitamin K but also tocopherylquinones. Owing to the difference in their reaction rates, vitamin K and tocopherylquinones may be distinguished from each other: extrapolation of the readings to zero time gives the vitamin K content of the sample and the final steady readings give the sum of both vitamin K and tocopherylquinones. Thus, the differences of the readings represent the amount of tocopherylquinones present. Preformed tocopherols may be converted into tocopherylquinones following oxidation with gold chloride (124). The difference of tocopherylquinone values in the gold chloride-treated and -untreated samples measures the tocopherol concentration in the original sample.

For *reagents, apparatus* and details of the intricate method as applied to vitamin K, see page 210. For determination of tocopherols and tocopherylquinones the following modification may be used.

Method.

The sample to be tested is dissolved in butanol and reduced over Raney's nickel catalyst in the presence of phenosafranine as the indicator. The specificity of the method can be increased by the use of the preliminary reductive treatment with Claisen's alkali as described for vitamin K. When the reduction over Raney's nickel catalyst is complete, the hydroquinones are titrated with a standard solution of 2, 6-dichloroindophenol in butanol. The diminution in blue color is a measure of the quinones present. Readings are taken in a photocolorimeter with the use of the appropriate filter (Filter 660 in the Evelyn photometer). Vitamin K hydroquinone reduces the indophenol instantaneously, whereas the reduction due to the tocopherylhydroquinones is completed only after 40-60 min. Serial readings are therefore taken. The reading at zero time gives the vitamin K content and the final steady reading gives the sum of concentrations of vitamin K and tocopherylquinones.

To apply this method to the determination of the tocopherols, it is only necessary to convert these compounds to the corresponding quinones. This is carried out as follows: Dissolve the sample, containing about 150-300 γ of the tocopherols, in 10 ml. of reagent butanol and add

0.5 ml. of a 20% aqueous solution of gold chloride ($AuCl_3.HCl.3H_2O$). Allow the solution to stand in the absence of light at room temperature for 30 min. Add 10 ml. of water and 30 ml. of technical hexane (b.p. 65-70° C.) and shake the mixture in a separatory funnel. Draw off the aqueous layer and wash the organic layer with two successive 10 ml. portions of water followed by 10 ml. of carbonate buffer (pH 9.7), and complete the washing with an additional 10 ml. of water. Draw off the organic layer, centrifuge it to remove precipitated gold oxides, and dry the extract superficially with anhydrous Na_2SO_4. Filter, and concentrate an aliquot of the extract to a small volume *in vacuo* under nitrogen. Add sufficient phenosafranine solution to give a final concentration of 0.5 γ of the indicator/ml., adjust to a convenient volume with butanol-acetate solution, and analyze as the original sample. Here again, preliminary reductive treatment with Claisen's alkali may increase the specificity of the method.

If applied to oils known to possess vitamin E activity, comparatively large samples (0.1-1.0 ml.) are analyzed directly for their tocopherylquinone content. Smaller samples of the oils (0.05-0.3 ml.) dissolved in butanol are oxidized with gold chloride and analyzed for their tocopherol content. The use of the large excess of gold chloride as recommended is particularly essential in work with oils or concentrates, since carotenoids and other substances are also oxidized by gold chloride (124).

Blood (serum, plasma) may be extracted with ethanol (refluxed for 5 min. with 9 volumes of ethanol) in the absence of light, the precipitated proteins further extracted twice with 5 volumes of petroleum ether *in vacuo* under nitrogen. The combined extracts are concentrated and the residue is taken up in petroleum ether, washed with a 1:1 solution of ethanol in water to remove extraneous colors and analyzed in the usual manner.

This colorimetric oxidation-reduction method measures the total tocopherols and tocopherylquinones, but does not distinguish between the biologically more potent α-tocopherol and the less active β-, and γ-, and δ-tocopherols.

D. CHEMICAL ASSAY OF THE INDIVIDUAL TOCOPHEROLS IN A MIXTURE OF THE α-, β-, γ-, AND δ-FORMS (126a)

Among the 4 known naturally occurring members of the vitamin E group only β-, γ-, and δ-tocopherols, but not α-tocopherol, form in alcoholic solution yellow nitroso derivatives on treatment with nitrous acid. This color reaction, first described by Scudi and Buhs (111) is the basis of a method for the specific determination of β-, γ- and δ-tocopherols in admixture, and in the presence of α-tocopherol. The latter is determined

by difference between total tocopherols and non-α-tocopherols. The total tocopherols may be measured by the Emmerie and Engel reaction, modified to take account of the very different behavior of δ-tocopherol from that of the α-, β-, and γ-forms (122).

The procedure for the assay of the individual, or of combined non-α-tocopherol is as follows (126a).

Reagents.

Glacial acetic acid.

Sodium nitrite (2 g./100 ml. of distilled water). The solution should be made fresh every few days, then be kept in the refrigerator and be warmed to room temperature before using.

Potassium hydroxide (20 g./100 ml. of distilled water).

Anhydrous sodium sulfate.

Skellysolve H.

Method.

All operations should be performed under subdued artificial light.

Five ml. of an absolute ethanol solution of the oil containing at least 0.25 mg. and not more than 1 mg. of non-α-tocopherols are placed in a 50 ml. glass-stoppered graduated cylinder. Exactly 0.2 ml. of glacial acetic acid are added and mixed, followed by exactly 3 ml. of sodium nitrite reagent from a fast delivery pipette. The mixture should be swirled vigorously for 5 sec. and left standing exactly 60 sec. Two ml. of KOH reagent are added, followed by 10 ml. of distilled water, a pinch of anhydrous Na_2SO_4 and exactly 12 ml. of Skellysolve H. The mixture should be stoppered, shaken vigorously for 30 sec. and left to settle. At least 10 ml. of the upper layer are transferred to an Evelyn photometer tube and read with Filter 400 against a blank which has been prepared in exactly the same fashion. Beckman spectrophotometer (at 410 mμ) may also be used. However, the Evelyn photometer is more sensitive, simpler and more convenient to use.

Calculation. For Evelyn photometer readings, $C = L_{400} \times K$, where C = mg. of non-α-tocopherols in the original 5 ml. of alcoholic solution and $L_{400} = 2 - \log G_{400}$.

The K values are determined with pure, natural (dextro) β-, γ- and δ-tocopherols.

The K values for γ- and δ-tocopherols are almost identical, that for β-tocopherol is slightly, but significantly, different. Thus, in an unknown mixture of all 3 non-α-tocopherols it is advisable and desirable to attempt a separate analysis of the 3 nitrosotocopherols. This may be carried out by chromatography (126a).

Chromatographic separation of nitrosotocopherols. At least 12 ml. of the Skellysolve extract of nitrosotocopherols (see above) are transferred into a glass-stoppered cylinder. An equal volume of distilled water and a pinch of anhydrous Na_2SO_4 are added. The flask is shaken for 30 sec. to wash out the small amount of ethanol which is dissolved in the Skellysolve H layer.

The water-washed Skellysolve H solution of the nitrosotocopherols is passed through a chromatograph column. The column 15 × 1.3 cm. is packed with $ZnCO_3$-Celite (70:30),[4] with a sintered glass disk set in the bottom to retain the adsorbent. The adsorbent should be added under suction in small portions and each one tamped down firmly.

Without allowing air to enter the column, 10 ml. of the water-washed Skellysolve H solution of the nitrosotocopherols are pipetted onto the column, through which pure Skellysolve H was previously passed (under suction). The solution of the nitrosotocopherols is allowed to run through the column almost completely. The last traces are washed through with several successive small (less than 1 ml.) quantities of Skellysolve H. The walls of the column are rinsed with a 5-10 ml. portion of Skellysolve H.

Without letting the column run dry, the chromatogram is developed with pure benzene. Washing is continued with benzene until the nitroso-γ- and nitroso-β-tocopherol rings (lowest and middle, respectively) have been washed through into separate receivers. The nitroso-δ-tocopherol ring (or rings) is eluted with 1:1 ether-ethanol and collected in one container. (Alternatively, the column can be sucked fairly dry and the rings separately extruded with a spatula, put immediately into a flask containing ethanol-ether, and these filtered through filter paper containing a layer of Celite to remove the adsorbent.)

All 3 eluates should be evaporated to dryness under a stream of nitrogen, taking care not to overheat the residue. Each residue may be taken up in 10.0 ml. of Skellysolve H, cooled to room temperature and transferred to Evelyn photometer. Reading (with Filter 400) and evaluation of the results is done as in the mixture, using the respective *K* values for nitroso-β-, γ-, and δ-tocopherols.

IV. Vitamin K

The various K vitamins (natural and synthetic) differ in their chemical composition, but all share the chemical characteristic of being a quinone or being easily oxidized to one. Most chemical methods proposed for the chemical determination of the K vitamins are based on oxidation-reduction titrations or on color reactions utilizing the quinone character of vitamin K. Owing to the fact that 2-methyl-1,4-naphthoquinone and closely related synthetic products have largely replaced the natural K-vitamins (or concentrates) in therapeutic and prophylactic usage, special attention is given in several chemical methods to the determination of 2-methyl-1,4-naphthoquinone and related synthetic products.

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[4] $ZnCO_3$, "purified, precipitated", obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Celite is diatomaceous earth obtained from the Johns-Manville Sales Corporation, Celite Division, 220 Delaware Ave., Buffalo, N. Y. The grade used is Celite 501.

Most of these methods are not specific enough to be applied to biological material, with their high content of various interfering substances, and their use is limited to relatively pure concentrates and mixtures such as are present in pharmaceutical preparations.

1. ASSAY METHODS FOR 2-METHYL-1,4-NAPHTHOQUINONE

Rosin, Rosenblum and Mack (127) have devised two methods of assay for the determination of 2-methyl-1,4-naphthoquinone which is known also under the proprietary name (accepted by the Americal Medical Association) of menadione. The methods are not specific and are to be used only for the drug or simple mixtures.

A. ROSIN'S METHODS

Method I of Rosin is an oxidation-reduction method. An accurately weighed quantity of 0.1-0.15 g. of 2-methyl-1,4-naphthoquinone, previously dried over H_2SO_4 for 3 hr., is placed in a flask, 10 cc. of glacial acetic acid added and followed by 10 cc. of 10% HCl. From mixtures (not containing interfering substances) 2-methy-1,4-naphthoquinone may be extracted with chloroform, the extract evaporated to dryness at room temperature and in subdued light by means of a current of air drawn through the flask. The residue is treated with glacial acetic acid and HCl as stated above. About 1 g. of zinc dust is added to the solution and the mixture allowed to stand in subdued light for 0.5 hr. with occasional agitation. The solution is then diluted with air-free water, quickly decanted through a pledget of cotton, the reduction flask and the cotton washed with three 10 cc. portions of air-free distilled water, and the filtrate promptly titrated with 0.1 *N* ceric sulfate, 0.1 cc. of *o*-phenanthroline T.S. being used as indicator.

Method II is a bromination method and applicable to the pure substance only and as such is of no special interest for biological purposes.

B. KOFLER'S COLORIMETRIC METHOD (128)

This method is based on the color reaction of 2-methyl-1,4-naphthoquinone with cyanoacetic acid ethyl ester (129).

2-Methyl-1,4-naphthoquinone or the dry residue of an ether extract prepared from samples containing 2-methyl-1,4-naphthoquinone is dissolved in equal parts of 95% alcohol and water. The evaporation of ether from the ether extract should be carried out carefully (2-methyl-1,4-naphthoquinone being volatile itself) at room temperature and at reduced pressure. For the development of color 1-3 drops (each) of cyanoacetic acid ester and ammonia (10%) are added to 5-12 ml. of the dilute alcohol solution. The ratio of the reagents to the test solution depends on the concentration of 2-methyl-1,4-naphthoquinone present in the sample to be tested. With higher concentration (50-300 γ) of 2-methyl-1,4-naphthoquinone, 1 drop (each) of the reagents 1 to 5 ml. of the test solution, and with lower concentration (10-50 γ) 3 drops each in 12 cc. represent the desired ratio. The mixtures are shaken for 2 min. and the color read after 10 min., either in a spectrophotometer or in a photoelectric colorimeter. Kofler recommends the use of filter S 57 for the Pulfrich Photometer. The necessary reference curve is prepared with the use of graded amounts of pure 2-methyl-1,4-naphthoquinone.

According to Kofler, this method may also be applied to urine and serum (plasma). Ten ml. of urine or serum (plasma) are required for the color reaction (with preceding ether extraction). Inasmuch as serum, and especially urine, may contain in various amounts substances which, when passing into the ether extract, may interfere with the final colorimetric reading, it is advisable to use special blanks as controls, which should be prepared in the same way as the original test samples, in equal volume and with the addition of ammonia but without the color reagent, cyanoacetic acid ester.

There is no further report on the precision and reproducibility of the method.

C. COLORIMETRIC TEST FOR VITAMIN K WITH SODIUM DIETHYL DITHIOCARBAMATE AND ALCOHOL ALKALI

Irreverre and Sullivan (130) claimed that the colorimetric test dedeveloped by them for vitamin K with sodium diethyl dithiocarbamate and alcohol alkali may also be used as a quantitative method for the determination of vitamin K. They have not stated, however, whether the method is specific and sensitive enough to be used in biological materials, and they have not indicated in their short report the precision achieved and expected. In consequence, the usefulness of the method for practical purpose has still to be demonstrated.

Reagents.

Ethanol, 95%.

Sodium alcoholate. (Dissolve 2 g. of sodium in 100 ml. of 95% ethanol.)

5% solution of sodium diethyl dithiocarbamate in 95% ethanol. (Sodium diethyl dithiocarbamate may be obtained from Eastman Kodak Co. If the substance is not pure, it should be recrystallized from warm 95% ethanol with decolorization by carboraffin. A 5% solution of the colorless purified material in 95% alcohol should be practically colorless. This reagent must be freshly made and will keep for only a day.)

The test is performed as follows: To 2 ml. of a 95% alcohol solution of the material to be tested, add 2 ml. of 5% sodium diethyl dithiocarbamate in 95% ethanol and 1 cc. of alcohol alkali. Under this condition, vitamin K gives a deep cobalt blue color, attaining its highest intensity in about 5 min. and fading slowly after 8 min. With the use of a photoelectric colorimeter and appropriate green filter (Irreverre and Sullivan used a Klett-Summerson photoelectric colorimeter and No. 54 green filter), the reaction is practically quantitative for vitamin K in pure solution, from a range of 0.01 mg/2 cc. to 1.0 mg./2 cc. Since the color is stable only for a few minutes the colorimetric readings must be taken every minute for 10 min. immediately following the addition of the last reagent. The highest reading is then used. The stability of the color changes with respect to concentration. At lower concentration the color is more stable than at the higher ones.

It should be added that this method constitutes a more sensitive modification of a colorimetric test for vitamin K with sodium alcoholate originally proposed by Dam, Karrer and their associates (131), with the additionally claimed advantage that it may be used quantitatively.

D. COLORIMETRIC OXIDATION-REDUCTION METHOD FOR THE DETERMINATION OF THE K VITAMINS BY SCUDI AND BUHS (125, 126)

The most ambitious and equally delicate method for the determination of the K vitamins, with the help of a colorimetric oxidation-reduction reaction, has been proposed and developed by Scudi and Buhs (125, 126). The method is based on the oxidation-reduction titrations reported by Trenner and Bacher (133); it involves a catalytic reduction of the quinone in butanol solution in the presence of phenosafranine as the indicator. The resulting vitamin hydroquinone is then treated with an excess of a butanol solution of 2,6-dichloroindophenol in the absence of air. The diminution in the color of the indophenol is a measure of the quinone originally present.

Reagents.

Butanol Acetate Solution. Five hundred mg. of reagent potassium acetate are dissolved in 50 ml. of water and the solution is diluted to 1 liter with acid-free *n*-butanol.

Phenosafranine. A stock solution containing 1 mg. of phenosafranine (Eastman, No. 1125)/ml. of water is prepared. One ml. of the aqueous solution is diluted to 100 ml. with *n*-butanol to give the reagent (10 γ/ml.).

Raney's Nickel. This is prepared according to Covert and Adkins (132). Three hundred g. of commercially available finely ground nickel-aluminum alloy is added slowly (2 or 3 hr.) to a solution of 300 g. of NaOH (80%) in 1200 ml. of distilled water, contained in a 4 l. beaker surrounded by ice. The mixture is then heated on a hot-plate for 4 hr. with occasional stirring at 115-120° C. A further 400 ml. of a 19% solution of NaOH is added and the mixture kept at 115-120° C. for about 3 hr. or until bubbles of hydrogen are no longer evolved, after which it is diluted to a volume of 3 l. The clear solution of sodium aluminate is decanted, and the nickel washed by decantation 6 times, and then alternately by suspension and by washing on a Buchner filter with distilled water until the filtrate is neutral to litmus. The final washing (133) is carried out with 95% ethanol containing some acetic acid. A batch of catalyst may be considered satisfactorily washed when the wash alcohol from it, after standing, gives a neutral reaction with bromthymol blue indicator. Finally all "fines" must be removed. This is accomplished by shaking the prepared nickel catalyst with a relatively large volume of 95% ethanol, allowing to settle for 1-2 min., and then quickly decanting off the liquid. This process is repeated a sufficient number of times until the residual catalyst settles off rapidly, leaving no appreciable cloud in the liquid phase. Final grading is made by washing the solid on a 400 mesh sieve with a stream of alcohol. The material remaining on the screen after this treatment is washed into a 15 cc. centrifuge tube and stored under alcohol ready for use. Such material is readily filtered through a cotton plug, giving a clear filtrate.

Petroleum ether (b.p. 60-80° C.).

2,6-Dichloroindophenol. A stock solution is prepared by shaking 50 mg. of the Eastman material (No. P-3463) for 15-20 min. in 100 ml. of butanol. The solution is filtered by suction through a dry filter paper. This stock solution, stored in a dark bottle in a refrigerator, is stable for at least 3 months. One ml. of this stock solution is diluted to approximately 50 cc. with the butanol-acetate solution. Ten ml. of this solution plus 5 ml. of the butanol-acetate solution gives an absorption of 85% in the Evelyn colorimeter with filter 660 (maximum absorption of 2,6-dichloroindophenol at 670 mμ), when pure butanol is used to indicate 100% transmission. The variable amounts of salts, water, *etc.*, in commercial samples of 2,6-dichloroindophenol make it impossible to state the exact dilution of the stock solution. Different samples of the indophenol require slightly different dilution, which may be followed colorimetrically up to the desired absorption of exactly 85%.

Claisen's Alkali. Fifty g. of KOH in 25 ml. of water diluted to 100 ml. with methanol.

Apparatus.

The apparatus shown diagramatically in Fig. 7 is constructed of glass with standard taper joints. It consists of two parts; a lower chamber A, in which the catalytic reduction is carried out, and an upper chamber B, in which the vitamin K hydroquinone partially reduces the standard indophenol reagent. Within limits, the dimensions of the apparatus are arbitrary. Scudi and Buhs recommend a construction to permit the convenient reduction of 7 ml. of solution in the lower chamber. Five ml. of the reduced solution can then be pumped into the upper chamber. The upper chamber (12 mm., outside diameter, tubing) was calibrated at 12 ml. These volumes may be reduced considerably.

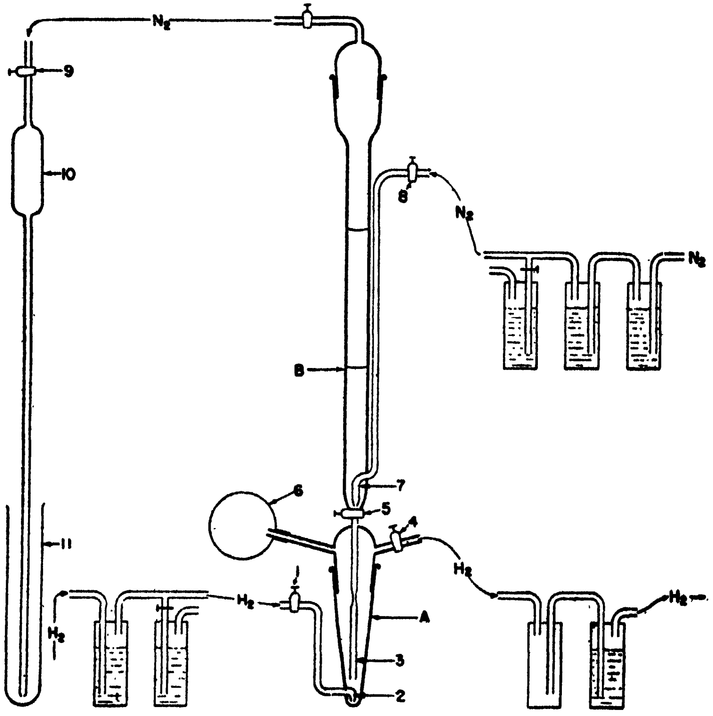

Fig. 7. Apparatus for the determination of the K vitamins (after Scudi and Buhs (125)).

In the presence of colored materials in the test sample, a preliminary reductive treatment of the sample with Claisen's alkali converts the colored concentrate to an essentially colorless solution and adds greatly to the specificity of the test, since only polysubstituted hydroquinones, which possess fat-soluble groupings, pass into the solution which is tested. The preliminary reduction with Claisen's alkali is carried out in a special apparatus shown diagrammatically in Fig. 8. Although the dimensions of the apparatus are more or less arbitrary, a total volume of 100 ml. of solution represents a satisfactory dimension.

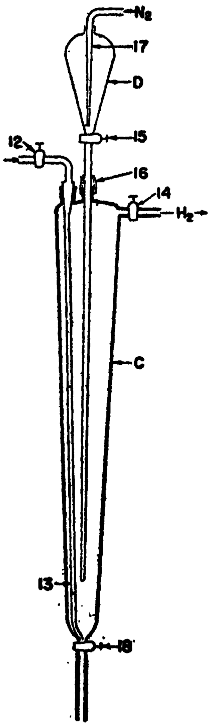

Fig. 8. Apparatus for the separation of the vitamin K hydroquinones from colored extracts by means of the reductive Claisen's alkali treatment (after Scudi and Buhs (125)).

Method.

Preparation of Extracts. Samples are dehydrated by refluxing for 3 hr. with alcohol. The solids, removed by filtration, are extracted in the dark for 48 to 96 hr. in a Soxhlet apparatus with petroleum ether (b.p. 60-80° C.). It should be emphasized that all steps performed during the analytical procedure are carried out with a minimum exposure to light. The combined alcohol-ether extracts are concentrated to dryness *in vacuo* under nitrogen, and the residue is taken up in a minimal volume of petroleum ether. After necessary adjustment of pH, the solution is diluted to contain 2.5-15 γ of vitamin K/ml.

The pH of the solution to be tested is important. An accumulation of organic acids in an extract gives erroneous results. The reduction does not proceed smoothly in acid solutions, and, furthermore, these acids convert the indophenol sodium salt, either entirely or in part, to the red-colored free indophenol. The pH of the petroleum ether solution can best be tested by mixing an aliquot of the sample with the indophenol reagent. If the unknown is acidic, a diminution in the blue color results. The pH of acid samples can be corrected as follows: A 10 ml. portion of a petroleum ether solution of the unknown is shaken with an equal volume of cold half-saturated baryta solution. The ether layer is washed with 5 ml. water, and again with 5 ml. of a 1 : 1 alcohol-water solution. The aqueous washes are extracted with 10 ml. of petroleum ether, and the combined ether extracts are dried over anhydrous Na_2SO_4. The ether extract is concentrated to dryness *in vacuo,* under nitrogen, and the residue is taken up in a minimal volume of petroleum ether.

Ten ml. of this solution, containing less than 2 g. of solids or oils, and 5 ml. of methanol are then submitted to reduction and treatment with Claisen's alkali. The solution, together with 10-15 mg. of powdered phenosafranine is placed in the lower chamber C (Fig. 8), and a charge of Raney's nickel (about the size of a pea, or larger, if the reduction is too slow) is added. The hydrogen is led through a methanol tower to prevent consequent evaporation losses before it is admitted through stopcock 12. The hydrogen is passed through inlet 13 at a rate sufficiently brisk to keep the catalyst in motion. The large amount of phenosafranine makes it relatively simple to follow the reduction in spite of the strong color of some concentrates. The reduced phenosafranine also protects the vitamin hydroquinone from accidental atmospheric oxidation. A leakage of air into the system, which vitiates the results, is readily detected by the return of the color of the phenosafranine.

After the reduction is complete, 15 ml. of Claisen's alkali are placed in the upper chamber D. Air is removed by bubbling nitrogen from inlet 17 through this solution—a process usually requiring about 10 min. The Claisen's alkali is then admitted through stopcock 15 and the contents of the lower chamber are mixed by readmitting the hydrogen stream through stopcock 12. The potassium salt of the vitamin hydroquinone is thus formed and remains in the alkaline phase.

The hydrogen supply is then cut off by closing cock 12. Cock 14 is left open during this operation. The system is protected from the entrance of air by connecting cock 14 with a trap such as shown connected to cock 4 in Fig. 7. The two phases are allowed to separate. The upper chamber D is then raised through the greased rubber collar 16 so that the petroleum ether can be removed by connecting an aspirator to the mouth of the upper chamber. This washing procedure, which serves to remove neutral fats, vitamin E, and ether-soluble chromogens, may be repeated as necessary.

After the petroleum ether is removed, 30 ml. of thoroughly air-free water are admitted through stockcock 15 to hydrolyze the potassium salt of the vitamin K hydroquinone. After the material is mixed for 5 min., 30 ml. of air-free petroleum ether are run through cock 15 to extract hydroquinone. After the material is mixed for 10 min. with hydrogen, the two phases are allowed to separate, and the highly colored, aqueous, alkaline, lower layer, now free of vitamin K, is drawn off through stopcock 18. The petroleum ether layer, which contains the hydroquinone, is then washed in the apparatus with 30 ml. of gas-free water which are subsequently removed by way of cock 18. The petroleum ether is run into a graduated cylinder and its volume is measured and the sample is dried over anhydrous Na_2SO_4. At this point, the hydroquinone is exposed for the first time to air oxidation. The small amount of phenosafranine in the petroleum ether becomes pink, but is adsorbed by the Na_2SO_4, leaving practically colorless solutions. The vitamin is transferred to butanol by adding butanol and distilling off the petroleum ether *in vacuo*. No pH adjustment is necessary following this procedure. This solution is then ready for analysis.

The entire apparatus, as shown in Fig. 7, is thoroughly cleansed with acetone and dried on a vacuum line. A small amount of the catalyst (about half the size of a pea) is placed in the bottom of the lower chamber. The sample if not colored is dissolved directly in the butanol-acetate reagent; if colored, it is first treated with Claisen's alkali as described and is finally also obtained in butanol solution. This solution, with phenosafranine solution added (0.5 cc. to 9.5 cc. of butanol-acetate), is placed in the lower chamber together with the catalyst. A cotton plug is placed in the constricted delivery tube 3 and the apparatus is set up as shown. With stopcocks 8, 5, and 4 open and cock 1 closed, the solution in the delivery tube 3 is replaced with nitrogen. Cocks 8 and 5 are closed, and 1 and 4 are opened.

Commercial hydrogen is passed through a spiral tower containing butanol to prevent evaporation losses. A water seal blow-off is placed in the line to prevent excessive pressures. The hydrogen is admitted through inlet 2 at a rate sufficiently brisk to keep the catalyst in motion.

During this reduction period the rubber bulb 6 is squeezed from time to time to expel the air within it. After all the quinone is reduced, the phenosafranine is reduced and its pink color disappears. The reduction is continued for an additional 5-10 min. to insure complete reduction.

While this reduction is in progress, 10 ml. of the standard indophenol reagent are pipetted into the upper chamber, and, as the reduction in the lower chamber approaches completion, the air in the indophenol solution is removed with a stream of nitrogen. This stream of commercial nitrogen is passed through a spiral tower containing alkaline hydrosulfite to remove traces of oxygen. The gas then passes through a butanol tower to saturate the gas. The gas train is by-passed with a butanol blow-off tower as a precautionary measure. The nitrogen is then admitted, at inlet 7, bubbled through the indophenol solution for 10 min., and it issues through the long-stemmed, uncalibrated pipette 10 which is placed in the (Evelyn) colorimeter tube 11, as shown in Fig. 7.

After the reduction is complete, stockcocks 1, 4, and 8 are closed. By means of the rubber bulb 6, the contents of the lower chamber are forced through the cotton filter (delivery tube 3) into the upper chamber as stopcock 5 is opened. When the volume reaches the 15 ml. mark, cock 5 is closed and nitrogen is readmitted through cock 8 for 1-2 min. to mix the contents of the upper chamber. Cocks 8 and 9 are then closed.

A deflated rubber bulb is placed on the end of the pipette, cock 9 remaining closed. The contents of the upper chamber are withdrawn by inserting the nitrogen-filled pipette into the chamber and slowly opening cock 9. When the solution is thus drawn up into the pipette, cock 9 is closed, and the rubber bulb is removed. The long stem of the pipette is then placed in the nitrogen-filled colorimeter tube, and the solution is run without splashing. Readings are taken in the colorimeter exactly 3 min. after the hydroquinone and the indophenol are mixed.

With respect to the specificity of the test, it was found (126) that certain concentrates, even after reductive treatment with Claisen's alkali, may contain extraneous substances which could simulate vitamin K in every respect except one; namely, they reduce the indophenol at a much lower rate, and are, consequently, easily detected in the course of the test. These substances were shown to possess the properties of the tocopherylquinones and their interference in the performance of the test was generally diminished by restricting the reading time to 3 min., in which time the reduction of the indophenol by the vitamin K hydroquinone is complete, whereas the reduction due to the tocopherylhydroquinones is only just beginning. However, as the relative amounts of the tocopherylquinones increase, the error becomes significant, and if the tocopheryl content is large, and the vitamin K content is small, then entirely erroneous results will be obtained. This error may be obviated through readings at repeated intervals after the first reading at 3 min., and by extrapolation to zero time (126).[5]

The test is applicable to solutions containing 2-10 γ of 2-methyl-1,4-naphthoquinone/cc. The colorimeter is calibrated against this material, since it is a crystalline solid and is more stable to light than vitamin K_1. A reference curve is established by the use of graded amounts of 2-methyl-1,4-naphthoquinone. The values are multiplied by 2.615 in order to convert them stoichiometrically to vitamin K_1.

The method is too delicate to offer a good and reliable substitute for the usual bioassay of the K vitamins in biological materials.

V. Thiamine

1. Historical Development

Most of the colorimetric methods proposed for the determination of thiamine depend on the reaction of the vitamin with a diazotized reagent. The few reactions which do not fall into this class may be mentioned first.

Lipman (134) pointed out that thiamine reacts with hydrosulfite to produce a very distinct green yellowish color, but this reaction was not developed into an analytical method. In similar brief notes, Tauber (135) described the reaction of thiamine with *p*-dimethylaminobenzaldehyde which, after evaporation, cooling, and addition of glacial acetic acid, yielded an intense brick red color, and Raybin (136) stated that thiamine reacts with alcoholic 2,6-dibromoquinonechloroimide to give an orange color extractable by chloroform.

The methods generally in use are based on the principle worked up on a quantitative basis by Koessler and Hanke (137), namely, the reaction with a diazotized reagent. Various reagents have been proposed. Kinnersley and Peters (138, 139) developed a method based on the reaction of thiamine, stabilized with formaldehyde, with diazotized sulfanilic acid in an alkaline medium. The use of this reagent was also studied by Meunier and Blancpain (140, 141). Willstaedt and Bárány (142) described the formation of an ether-soluble azo dye by the reaction of thiamine with diazotized 2,4-dichloroaniline in an alkaline medium. Marenzi (143) stated that thiamine reacted with diazotized *p*-nitro-

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[5] With appropriate modifications the same procedure used for the determination of the K vitamins may be applicable to the quantitative determination of the tocopherols, after oxidation to the corresponding quinones. See page 204.

TABLE III

Comparison of Results by the Simplified Colorimetric and Thiochrome Procedures for the Determination of Thiamine[a]

Sample	Thiamine Values Found		$\frac{\text{Color.} - \text{Thiochrome}}{\text{Thiochrome}} \times 100$
	Colorimetric method	Thiochrome method	
	γ/g.	*γ/g.*	*per cent*
White flour	0.99	1.00	— 1.0
Enriched flour A	4.82	5.00	— 3.6
B	5.52	4.40	25.4
C	5.52	6.04	— 8.6
D	6.88	6.85	0.4
Whole wheat flour A	4.88	4.50	8.5
B	4.93	5.05	— 2.4
Enriched bread A, air-dried	3.92	3.87	1.3
B, air-dried	4.38	4.40	— 0.5
Bun, air-dried	0.62	0.73	— 12.3
Doughnut, air-dried	1.08	1.11	— 2.7
Enriched doughnut mix	4.69	4.54	3.3
Barley	3.79	3.50	8.3
Malted barley	3.62	3.64	— 0.5
Malt extract syrup	3.67	3.38	8.6
Dried malt extract	4.31	3.94	9.4
Enriched graham cracker	4.95	3.31	49.6
White rice	1.05	0.99	6.1
Brown rice	5.53	4.69	17.9
Wheat germ	29	26	11.5
Rice bran concentrate	148	144	2.8
Dried yeast powder	711	714	— 0.4
Crystalline Thiamine A[b] in %	95.0	94.0	1.1
" " B " "	101.8	99.6	2.2
" " C " "	90.2	89.2	1.1
			Av. 5.0%

[a] From Hochberg, Melnick and Oser (157).

[b] Dried over phosphorus pentoxide.

aniline in alkaline medium to form a red derivative extractable with benzene. Kirch and Bergeim (144, 145) worked up a method based on the reaction of thiamine with diazotized ethyl-*p*-aminobenzoate and extraction of the colored derivative with isoamyl alcohol.

The most successful reagent, diazotized *p*-aminoacetophenone, was first described by Prebluda and McCollum (146). In alkaline solutions, the reagent forms a red color, which is insoluble in water. Melnick and Field (147) described techniques for the extraction of the red pigment with xylene and purification and concentration of thiamine by adsorption on permutit (zeolite, Decalso). This reaction was developed into a satisfactory quantitative method through the further investigations of

Prebluda and McCollum (148), Melnick and Field (149-151), Emmett and coworkers (152, 153), Auerbach (154), Platt and Glock (155), Sealock and Goodland (156), and others. The recent paper of Hochberg, Melnick and Oser (157), describing the simplified colorimetric determination of thiamine in cereal products, represents the present status of this colorimetric thiamine method.

2. METHODS OF ANALYSIS

The method presented here follows the procedure described by Hochberg, Melnick and Oser (157), except for minor modifications.

Reagents.

Sulfuric Acid Solution. 0.1 *N.*

Sodium Acetate Solution. 2.5 *M.*

Phosphatase Preparation. Clarase (Takamine Laboratories, Clifton, N. J.).

Decalso. 40-60 mesh Decalso (Permutit Co., New York City). Care should be taken to obtain the proper mesh. The synthetic Decalso used for adsorption is prepared in bulk by stirring with four 10 volume portions of 3% acetic acid for 10 min. each. Between the second and third acid wash, a 15 min. treatment with 5 volumes of 25% KCl is introduced. The Decalso is washed thoroughly with water, alcohol, and ether, dried in air, and stored in a sealed bottle.

Salt Solution. 25% KCl in 0.1 *N* HCl.

Standard Thiamine Solution. Four γ of anhydrous, U.S.P. thiamine hydrochloride/ml. of 0.1 *N* HCl. This solution is stored in the refrigerator, and is stable.

p-Aminoacetophenone Solution. 6.35 g. *p*-aminoacetophenone are dissolved in 90 ml. of concentrated HCl and diluted to 1 l. with distilled water. The solution is stored in an amber bottle.

Sodium Nitrite Solution. 22.5 g. of $NaNO_2$ are dissolved in sufficient distilled water to make 500 ml. This solution is stored in the refrigerator and is stable for at least 3 months.

Sodium Hydroxide-Sodium Bicarbonate Solution. Forty g. of NaOH are dissolved in 1500 ml. of distilled water, 57.6 g. of $NaHCO_3$ added, and sufficient distilled water to bring the volume to 2 l.

Diazotized p-Aminoacetophenone Solution. Five ml. of the *p*-aminoacetophenone solution are pipetted into a 50 ml. graduate surrounded with chopped ice and provided with a stirrer; 5 ml. of the $NaNO_2$ solution are added slowly and the resulting solution stirred for 10 min. Then 20 ml. of the nitrite solution are again added slowly and the stirring continued for an additional 30 min. The temperature of diazotization should not exceed 5° C. This solution should be used within 24 hr. after preparation; when not in use, it should be kept below 5° C.

Thiamine Reagent. Twenty ml. of the above diazonium salt solution are added with stirring to 274 ml. of the NaOH-$NaHCO_3$ solution. The reagent is ready for use when the initial purple color changes to a pale yellow, which usually takes 5-20 min. This reagent is prepared immediately before use and only as much as can be used within 10 min. should be prepared at any one time.

Alcohol-Phenol Solution. 15.6 g. of phenol are dissolved in sufficient 95% alcohol to make 2 l. This solution is stored in an amber bottle.

Sodium Hydroxide Solutions. 5.0 *N* and 1.0 *N*.

Thymol Blue Indicator. 1% solution in alcohol.

Xylene. C.P.

Apparatus.

The apparatus for the concentration of the extract and removal of interfering substances is shown in Fig. 9. A glass funnel is connected to a condenser by means of a short piece of rubber tubing or, preferably, by a glass joint. The condenser, which is connected to a steam outlet, is similarly joined to a tube having an internal diameter of 8 mm. for a length of 4.5 in., followed by a constricted portion about 4 mm. in diameter. A plug of glass wool is placed at the top of the constriction, and

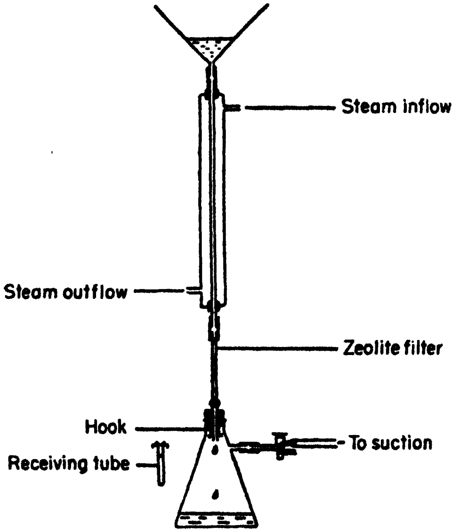

Fig. 9. Simplified apparatus for adsorption and elution of thiamine (after Hochberg *et al.* (157)).

exactly 3.0 g. of the treated Decalso added to the tube. The tube containing the adsorbent is connected to a suction flask by means of a rubber stopper equipped with a small hook which permits hanging a receiving test tube below the Decalso column.

Procedure.

A sample containing between 30 and 100 γ of thiamine, but not larger than 20 g., is weighed into a 250 ml. volumetric flask, and 150 ml. of 0.1 N H_2SO_4 are added. The suspension is refluxed at 100° C. for 30-45 min. The mixture is cooled and 10 ml. of 2.5 M sodium acetate are added to adjust the pH to 4.5. Three per cent by weight of clarase is added and the suspension is incubated for at least 3 hr., preferably overnight, at 45° C., then cooled to room temperature and brought to 250 ml. with water. The insoluble material is removed by centrifugation or filtration through No. 40 Whatman paper.

An aliquot of the clear extract, containing 30-100 γ of thiamine is passed through the Decalso column at room temperature without suction. Steam is then passed through the outside jacket, and 30 ml. of water are poured on the column. This is allowed to heat for ½ min., and is then drawn through with rapid suction to heat the Decalso. The receiving tube is attached and the thiamine is eluted immediately by passing 20 ml. of the KCl solution in 3 portions (10, 5, 5 ml.) down the hot wall of the condenser. Each portion of the eluate is collected in the receiving tube at the rate of approximately 1 drop/2 sec., and the last few drops are drawn through by suction before the next portion is added. The Decalso is washed with three 100 ml. portions of distilled water under full suction while steam passes through the jacket. Between the second and third hot water wash, 25 ml. of 3% acetic acid are run through the hot column with suction. The column is finally cooled to room temperature by running 100 ml. of distilled water through with the steam turned off. The apparatus is then ready for the next extract.

Standard. Sufficient working standard thiamine solution is prepared so that at least two aliquots are available for passage through Decalso and colorimetry. Twenty-five ml. of the stock standard described above are pipetted into a 100 ml. volumetric flask, followed by 1.3 ml. of 2.5 M sodium acetate for adjustment to pH 4.5, and diluted to volume. Forty ml. aliquots (containing 40 γ of thiamine) are then taken through Decalso in the same fashion as the unknowns, preferably at the beginning and end of a series.

The eluate is transferred to a 100 ml. volumetric flask; 20 ml. of the alcohol-phenol reagent are passed through the receiving tube as a wash and added to the flask followed by 2 drops of the thymol blue indicator. First, 5 N NaOH, then 1 N NaOH is added dropwise until the first distinct blue color is produced. Immediately 35 ml. of the freshly prepared thiamine reagent are added, the solutions are mixed and allowed to stand in the dark at room temperature overnight. Then 12 ml. of xylene are added and the flask shaken vigorously for 3 min. to extract the pigment formed by the reaction between the vitamin and the reagent. The mixture is poured into a 100 ml. centrifuge tube and the layers separated by a short centrifugation. The xylene layer is then transferred to the absorption cell of a photoelectric colorimeter (520 mμ filter) by means of a 10 ml. pipette connected by a long rubber tube to a syringe for gentle controlled suction. The colorimeter is set to 100 with pure xylene and the photometric densities of unknown and standard solutions determined.

Calculation for Evelyn photoelectric colorimeter: If L_s *and* L_u = optical density ($2 - \log g$) of standard and unknown, respectively, $C_s = \gamma$ of thiamine in aliquot of standard put in column.

The constant for each standard is calculated according to $K = \frac{L_s}{C_s}$ and the average K obtained.

Then $\frac{Lu}{Kav.} \times$ dilution factor = γ of thiamine in sample.

Notes on the Procedure.

a). This method has been applied to a variety of natural materials and pharmaceuticals (157) as shown in Table III. It may be seen that, on a comparative basis, the colorimetric method yields results which average 5% higher than the corresponding thiochrome values. The list of products may be extended to include any materials with sufficient potency (at least 2 γ/g.).

b). Visual means of measuring color intensities may be used with little loss of accuracy.

c). Acid extraction of samples containing iron in the form of ferrum reductum has been found to cause significant destruction of thiamine, the effect being particularly marked in the case of finely powdered materials such as flour premixes or corn meal premixes (158, 158a). This is believed to be due to a reduction of thiamine (*cf.* 134) to a form which is not measurable by either the thiochrome or the colorimetric method. This destruction of thiamine can be avoided by room temperature extraction with acetate buffer at pH 6.0 if the sample is of such a nature that simple shaking will insure complete extraction of thiamine. If hot extraction is necessary, it must be carried out at a low pH to avoid thermal destruction of thiamine (159). Hot acid extraction can be safely used in the presence of ferrum reductum if an acid solution of cystine is added initially to the sample. The amount of cystine to be added should be at least 10 times the weight of the ferrum reductum present in the sample taken for analysis. The cystine solution is prepared by dissolving cystine in concentrated HCl and diluting to the desired level.

d). Sealock and Goodland (156) reported a number of substances which interfere with the reaction between thiamine and *p*-aminoacetophenone. Ågren (160) found that thiamine is reduced by several substances, including ascorbic acid, cysteine, sodium hyposulfite and a phenol-like compound present in aspen leaves. Only in the case of sodium hyposulfite could the colorimetric method be applied, whereas the thiochrome method was applicable in all cases.

e). Van der Mijll Dekker (161) recommended the use of reagents of high purity. This author confirmed the observation of Melnick and Field (150) that, if the addition of *p*-aminoacetophenone produces a precipitate, some of the color is adsorbed. The precipitate should be dissolved by adding 20% H_2SO_4 before extraction of the color with xylene. When the solution is made alkaline and reshaken, non-specific colors are returned to the water layer, leaving the red dye in the xylene layer.

VI. Niacin and Niacinamide

1. HISTORICAL DEVELOPMENT

Following the demonstration by Elvehjem, Madden, Strong and Woolley (162, 163) that niacin (nicotinic acid) cures canine blacktongue, and the successful cure of human pellagra with niacin, first reported by Spies, Cooper, and Blankenhorn (164), numerous studies were made of the analytical chemistry and biological activity of this and related compounds. It was established that the only ones showing definite biological activity are niacin, niacinamide, and those compounds which are capable of oxidative or hydrolytic conversion to these substances in the body (165). Comprehensive reviews of the biological considerations are available (165, 166).

Various color reactions have been proposed for the determination of niacin and niacinamide. The reaction with 2,4-dinitrochlorobenzene, first suggested by Vongerichten (167) was used by Karrer and Keller (168) and Vilter, Spies, and Mathews (169) in the development of quantitative methods. Daroga (170) proposed the reaction of niacin, precipitated by phosphomolybdic acid and dissolved in alkali, with $SnCl_2$ to produce a blue color. Teeri and Shimer (171) developed a method with *m*-phenylenediamine and HCl. Goodyear and Murphy (172) proposed and studied a method for niacinamide based on the Hoffman rearrangement of the amide to yield 3-aminopyridine which is then diazotized and coupled with N-(1-naphthyl)-ethylenediamine to yield a red dye.

The more common methods are based on the reaction of α- or γ-unsubstituted pyridine derivatives (*cf.* 165) with cyanogen bromide and an aromatic amine to give a color, as developed by Koenig (173). Various aromatic amines have been used for the determination of niacin: aniline was chosen by Swaminathan (174), Kringstad and Naess (177), Melnick and coworkers (178-180), Lamb (181), Waisman and Elvehjem (182), and others; *p*-aminoacetophenone was preferred by Harris and Raymond (183), Arnold, Schreffler and Lipsius (184), Jones (185), Hale, Davis and Baldwin (186), James, Norris and Wokes (187), and others. The use of *p*-methylaminophenol sulfate (metol, Photol) as the aromatic amine in the Koenig reaction was first suggested by Bandier and Hald (188), and investigated extensively by Bandier (189, 190). While the reaction was satisfactory for pure solutions, difficulties were encountered when the analysis of biological or natural materials was attempted. These difficulties were encountered, regardless of the particular aromatic amine employed in the colorimetric reaction, and arise from the following problems: 1) the mode of extraction of natural materials to release niacin and to convert other biological forms of the vitamin into niacin; 2) purification of the hydrolyzate to remove interfering pigments and yield as colorless an extract for colorimetry as possible; 3) manner of setting up blanks to correct for irrelevant colors, either preformed in the extract or produced by the various reagents with substances other than niacin; 4) use of an increment procedure or a calibration constant for calculation of the concentration of niacin from the intensity of color measured.

a. Extraction. As pointed out by Elvehjem and Teply (165), a number of compounds related to niacin must be taken into account in the analysis of natural materials for this vitamin. Niacinamide is the most prevalent compound and has biological activity equal to niacin. The sources of niacinamide in biological materials are coenzyme I and coenzyme II, which undergo hydrolytic cleavage to release niacinamide. At least two other niacin derivatives must be considered in any analytical scheme: nicotinuric acid, which is active, and trigonelline, which is inactive. These are of special importance in urine analysis.

Bandier (190) pointed out that niacin, niacinamide, and nicotinuric acid each yields a color, but that the intensities of the colors produced by equimolar amounts of these compounds are quite different. He therefore proposed that these compounds be transformed into niacin before colorimetry. He recommended alkaline hydrolysis, since he found that the coenzymes and niacinamide are hydrolyzed to niacin by 1 *N* acid or alkali, but nicotinuric acid is not susceptible to this moderate acid hydrolysis. This mode of hydrolysis was used by other workers (185, 186). Perlzweig, Levy and Sarett (191) confirmed that nicotinuric acid is converted to niacin by mild alkaline hydrolysis, but pointed out that variable amounts of trigonelline are changed to a compound which reacts like niacin. To avoid this conversion of a biologically inactive form, which would introduce variable errors into the determination, these authors (191) proposed hydrolysis with concentrated HCl and HNO_3 for the conversion of niacinamide, coenzymes, and nicotinuric acid, since nicotinuric acid is hydrolyzed in strong (5 *N*) acid. Swaminathan (175), on the other hand, has carried out the hydrolysis in very dilute alkali to avoid conversion of trigonelline.

To convert trigonelline to a chromogenic form in a reproducible fashion, Perlzweig *et al.* recommended the treatment of a portion of the acid-hydrolyzed extract with 6 *N* KOH and urea. In this way, it became possible to determine separately the biologically active and inactive fractions. Dann and Handler (192), Waisman and Elvehjem (182), and Melnick (179), recommended the use of concentrated HCl to convert the biologically active compounds into niacin. Melnick (179) found that mild aqueous extraction gave the lowest niacin values and predigestion with enzymes slightly higher results, while maximal values, more than double the former, were obtained by acid and alkaline hydrolysis. Acid hydrolysis was preferred to avoid conversion of trigonelline. Cheldelin and Williams (193) carried out an extensive study of the various methods proposed for the extraction of niacin from animal and plant tissues and found that enzymatic digestion or acid or alkaline extraction gave the same niacin values for meats and milk, but that enzyme treatment gave consistently lower results for cereals. The increase due to alkaline hydrolysis was attributed in part to the conversion of trigonelline, while the higher values by acid hydrolysis were considered as evidence for a niacin precursor other than trigonelline but otherwise unidentified. Wang and Kodicek (194) stated that short digestion with 1 *N* NaOH is preferable to other hydrolytic treatments since trigonelline is not converted under these conditions, while the hydrolysis of nicotinuric acid is both more quantitative and more reliable. On the other hand, Steele, in the 1945 collaborative study of the American Association of Cereal Chemists (195), used H_2SO_4 as the only hydrolytic agent, while hydrolysis with concentrated HCl, preceded by autoclaving in water, was preferred by Melnick, Oser and Siegel (180).

A different approach to the problem has recently been provided by Friedemann and Frazier (196), who pointed out that all these methods of acid or alkaline hydrolysis have the undesirable feature of causing extensive decomposition of sugars and proteins, producing nonspecific, amine-reacting substances and extraneous colors. They (196) showed that digestion with a suspension of $Ca(OH)_2$ brought about the same degree of conversion of derivatives to niacin as 1 *N* NaOH (*i.e.*, complete hydrolysis of coenzymes, niacinamide and nicotinuric acid, no conversion of the inactive trigonelline), but with far less production of interfering substances.

b. Purification of Extract. The problem of purification of the extract is 2-fold. The removal of interfering pigments is needed since the presence of non-niacin colors in the final extract makes the accurate determination of the color formed by niacin more difficult or even impossible. Secondly, it is important to establish conditions of utmost specificity for the colorimetric reaction, eliminating as far as possible those pyridine derivatives which react similarly to the vitamin. Bandier (189, 190) found that the addition of KH_2PO_4 before colorimetry minimized the reaction of the color reagents with other pyridine derivatives. To remove other interfering pigments, he suggested precipitation of these pigments by treatment of the extract with an excess of acetone; the use of acetone was also proposed by Jones (185) and Montañes del Oro (197). Hale, Davis and Baldwin (186) recommended ethyl alcohol for the same purpose. Arnold, Schreffler and Lipsius (184) developed a biphasic method based on the extraction of niacin with ethyl acetate, leaving the interfering substances behind in the aqueous layer. A different principle was employed by Vilter *et al.* (169), Waisman and Elvehjem (182), and Melnick *et al.* (180), who proposed the use of charcoal to adsorb interfering pigments. It was later found, however, that charcoal also adsorbed variable quantities of niacin, causing losses of the vitamin during purification of the extract (185, 191, 192). Purification of the extract by adsorption-elution appeared nonetheless promising, and a quantitative procedure was developed by Perlzweig, Levy and Sarett (191), who proposed adsorption on Lloyd's reagent followed by treatment with $Pb(NO_3)_2$ to separate niacin from interfering compounds. Dann and Handler (192) applied the method to the estimation of niacin in animal tissues, emphasizing the importance of decolorizing the final extract as much as possible. This decolorization procedure has been adopted in the collaborative studies of niacin methods of the American Association of Cereal Chemists (179, 195) and in the method of James *et al.* (187). In their recent investigation of the colorimetric method, Friedemann and Frazier (196) proposed $Zn(OH)_2$ precipitation following digestion with $Ca(OH)_2$ for hydrolysis and decolorization; these authors stated, however, that after hydrolysis with conc. HCl, the decolorization procedure developed by Perlzweig and coworkers was satisfactory. Swaminathan (175-176) also utilized $Zn(OH)_2$, but James *et al.* (187) reported it to be no better than Lloyd's reagent in the analysis of cereals and other foods.

c. Estimation of the Blank. The third problem inherent in the colorimetric method revolves around the procedure to be followed in estimating the blank, *i.e.*, non-niacin colors. At first the solution was sought in a simple color blank. Bandier and Hald (188) proposed a set-up in which equal aliquots of the final extract were placed together with the buffer in 2 tubes, one to be treated with CNBr and metol, the other to be diluted to the same level with water. The blanks were read against a tube of distilled water, the other tubes against a reagent blank composed of water, buffer, CNBr, and metol. This procedure was widely adopted either in its original form (191, 192), or with slight modifications due to different experimental conditions (179, 181, 186, 187, 195), and has been incorporated in 2 methods described in the "Methods" section. It has, however, been pointed out by Wang and Kodicek (194) that this blank correction may be too low with certain extracts, since it is based on the assumption that a purified final extract contains no interfering substances which may react with the reagents to give a color, an assumption which does not always hold true. A second approach to the problem was the introduction of an "amine blank," *i.e.*, the only difference between the unknown tube and the blank tube was the absence from the latter of CNBr. This suggestion was made by Harris and Raymond (183), and has been followed by Arnold *et al.* (184) and Jones (185). Conversely, Bandier (198) proposed a CNBr blank in which only the amine was omitted from the reaction mixture. Objections to both the amine and CNBr blanks as the sole measure of non-niacin color have been advanced on theoretical and experimental grounds by Wang and Kodicek (194), and Friedemann and Frazier (196). Wang and Kodicek, working with *p*-aminoacetophenone, proposed an "acid blank" which was based on the fact that CNBr and niacin do not react in strongly acid solution. With this amine, the usual order of addition of the reagents is: sample, CNBr, *p*-aminoacetophenone, HCl; by simply adding the acid before CNBr, a satisfactory blank was obtained. Friedemann and Frazier, on the other hand, suggested that 4 tubes be set up as follows: 1) sample + CNBr + metol, to give total color; 2) sample + amine, to give the amine blank which is a measure of amine-reactive substances; 3) sample + CNBr, to give the CNBr blank which is a measure of CNBr-reactive substances; and 4) sample + H_2O, to give the color blank which is a measure of nonreactive residual color. All 4 tubes, read against their respective reagent blanks, are used in the calculations. It is apparent that the choice of a suitable blank is still a subject of considerable experimentation and discussion. The blank given in the "Methods" is the first described above, and is presented on the basis that its usage has led to values for a wide variety of foods which are in good agreement with independent microbiological assays (*cf.* note 9).

d. Method of Calculation (Increment Procedure). The fourth problem in niacin methodology concerns the use of an increment procedure as compared to a calibration constant for the establishment of a relationship between color intensity and concentration of the vitamin. Melnick (179, 180) used the increment method with the idea that this internal standard provides automatic correction for the variety of factors which influence the color reaction. He added a known increment of niacin to one of two equal aliquots of the extract prior to colorimetry and calculated the concentration of niacin in the unknown from the response due to the added amount of the vitamin. Waisman and Elvehjem (182), Hale, Davis and Baldwin (186), Wang and Kodicek (194), and Steele (195), followed this method of using the increment procedure. Rather than rely on a single increment, Harris and Raymond (183) set up 3 equal aliquots of extracts with graded amounts of added niacin, plotting the readings against concentration of added niacin; the straight line thus obtained was extrapolated back to zero concentration of added niacin to obtain the amount of niacin in the sample. Arnold *et al.* (184), Jones (185), and Lamb (181), used essentially the same principle. James *et al.* (187) set up increments at 2 different niacin levels.

Dann and Handler (192) have argued against the use of an increment on the grounds that "there is no way of compensating for new absorption produced by the interaction of the reagents with the pigment," but that the only remedy lies in adequate removal of interfering substances. Friedemann and Frazier (196) have advanced similar arguments, and attempt to resolve the difficulties by use of a more comprehensive series of blank measurements.

In view of these conflicting viewpoints both the calibration constant and the increment procedures are presented in detail in the "Methods" section.

2. METHODS OF ANALYSIS

Three procedures are presented in detail. The first, employing metol as the aromatic amine and relying on calculation by calibration constant, is based on the method of Perlzweig, Levy and Sarett (191), which was extended by Dann and Handler (192). The second, making use of aniline and the increment procedure, follows the procedure of Melnick in the 1942 collaborative studies of the American Association of Cereal Chemists (179). The third, utilizing *p*-aminoacetophenone, is the method recommended by the British workers, James, Norris and Wokes (187), who also compared the sensitivity and rate of formation and fading of the color in the reaction of nicotinic acid with metol, aniline, procaine, and *p*-aminoacetophenone. The optical density produced by metol was only a fifth of that produced by *p*-aminoacetophenone, less than a third of that produced by procaine, and less than half of that produced by aniline. The results given by metol were more variable, the coefficients of variation of a single observation being 11.9 as compared with 5.6 for aniline, 3.8 for procaine, and 2.4 for *p*-aminoacetophenone.

A. METOL PROCEDURE

After Dann and Handler (192)

a. Reagents.

Hydrochloric acid, C.P. concentrated.

Sodium hydroxide (50%), equal weights of NaOH (C.P.) and water.

Sodium hydroxide, 0.5 *N* aqueous.

Sulfuric acid, 0.2 *N* aqueous solution.

Lloyd's reagent.

Lead nitrate, anhydrous, powdered.

Phosphoric acid solutions, 20% and 4%.

Potassium phosphate, K_3PO_4, anhydrous, powdered.

Phenolphthalein, 1% in 95% alcohol.

Potassium dihydrogen phosphate, KH_2PO_4, 10% aqueous solution.

Cyanogen bromide, 3% aqueous solution. Eastman Kodak crystals are weighed directly (under the hood) into a 250 ml. glass-stoppered amber reagent bottle containing 25 ml. of water. The volume of water needed to make a 3% solution is then added. This solution may be stable for periods up to 5 months in the refrigerator. (Do not inhale—strong lachrymator.)

Metol, 5% aqueous solution. This solution is made up at that point of the colorimetry where the tubes are being brought to the required temperature of 75-80° C. before addition of the cyanogen bromide. The weighed metol (Merck Photol) is introduced into a glass-stoppered graduated cylinder and the solution brought to the desired volume with distilled water; (*e.g.,* if 15 tubes are to be colorized, 8 g. of metol are dissolved in enough water to give 160 ml. of solution). The cylinder is placed in a shaking machine and left to shake until the tubes are ready for the addition of the metol. At that time, the reagent should be crystal-clear and colorless. If a particular batch of metol gives a solution which darkens too quickly, it should be discarded.

Niacin Stock Standard (0.025 g.-%). 0.2500 g. of dried Niacin (Nicotinic Acid) U.S.P. Reference Standard is weighed accurately into a 1 l.

volumetric flask and diluted to the mark with distilled water. A few drops of chloroform are added as a preservative. The stock standard is stable for at least a year in the refrigerator.

Niacin Working Standard (0.500 mg.-%). Made up daily by diluting exactly 5 ml. of stock standard to 250 ml. with distilled water.

Accutint pH papers No. 10 (pH 0.0-1.2) and No. 70 (3.9-5.4) or equivalent pH indicator papers.

Hydrion B pH papers.

b. Apparatus.

Photoelectric colorimeter with filter at 400 mμ.

Constant temperature bath.

Adsorption tubes. 27 × 135 mm., round-bottomed, wide-mouthed, heavy glass centrifuge tubes with a capacity of 55-60 ml., and calibrated at a volume of 26.2 ml.

Interchangeable *colorimeter tubes* and colorimeter tube *racks.*

c. Procedure.

The potency relationships and colorimetry in this section are adapted to the Evelyn photoelectric colorimeter; similar conditions may easily be set up for other instruments.

1. Preparation of Sample and Hydrolysis. The final extract before colorimetry should contain 3-4 γ/ml. for optimum color development. Since adsorption on, and elution from, Lloyd's reagent concentrates the extract from 50 to 25 ml., the extract should contain 1.5-2 γ/ml. before adsorption. A representative sample containing at least 150 γ is weighed into a suitable volumetric flask (the smallest suitable volume is 100 ml., but in this case not more than 5 g. of sample should be taken (*cf.* 192)). Enough water is added to bring the total volume to three-fifths of the volume of the flask; a volume of conc. HCl equal to one-fifth of the volume of the flask is added (*e.g.,* sample weighed into 500 ml. volumetric flask, volume brought to 300 ml. with water, 100 ml. of conc. HCl added), so that the mixture is in 3 *N* acid at this point. A glass bulb is placed in the mouth of the flask to minimize loss of HCl and the flask is placed in a boiling water bath. Hydrolysis is allowed to proceed, with occasional swirling, until all solid particles are completely broken down, but not less than 1-1.5 hr. When the solution appears homogeneous, the flask is removed from the water bath and left to cool. Fifty per cent aqueous NaOH is added in the approximate ratio of 0.55 ml./ml. conc. HCl used previously; the pH of the solution should be between 0.5 and 1.0 (check with Accutint pH paper No. 10). During this addition, the flask is continuously cooled in a cold water bath. After dilution to the mark and thorough mixing, the solution is filtered through Whatman

No. 40 filter paper, the first fourth of the filtrate being discarded because of possible adsorption of niacin on the filter paper.

2. Decolorization of the Extract. Fifty ml. of the clear filtrate are pipetted into the adsorption tube containing 2.0 g. of Lloyd's reagent. The tubes are stoppered and shaken vigorously for 2 min., centrifuged for 5-10 min. and the supernatant discarded. The Lloyd's reagent is washed by shaking gently with about 25 ml. of 0.2 *N* H_2SO_4, centrifuged, and the washings discarded. 15.2 ml. of 0.5 *N* NaOH (7.6 m.Eq.) are added to the tube, taking care to wash down the sides of the tube, and the contents made up very carefully to the 26.2 ml. graduation with water (the volume of the liquid is then exactly 25 ml.). After being shaken vigorously for 2 min., the tube is centrifuged and the colored supernatant poured into a 50 ml. centrifuge tube (Rockefeller type) containing 1.0 g. of finely powdered $Pb(NO_3)_2$. This amount of $Pb(NO_3)_2$ is usually sufficient to bring the pH of the solution definitely to the acid side (pH 6). The pH is checked with Hydrion B paper. It may also be observed that flocculation sets in when the pH becomes less than 6.5-7.0. This may also be taken as an indication that enough $Pb(NO_3)_2$ has been added. If necessary, a small additional quantity of $Pb(NO_3)_2$ is added to bring about flocculation and an acid color on Hydrion B paper. The mixture is then centrifuged for 5 min. and the colorless or slightly colored supernatant poured off into another 50 ml. centrifuge tube. To remove the lead remaining in solution proceed as follows. To the clear supernatant are added 1 drop of 1% phenolphthalein and a sufficient quantity of solid, finely powered K_3PO_4 to produce a stable pink indicator color. The solution is centrifuged for 5 min. and the clear supernatant poured off into a 50 ml. beaker. The pH of the solution is brought to 4.5 (4.3-4.7 is sufficiently close) with 20% H_3PO_4 (1-2 drops) and finally with 4% H_3PO_4 (the dilution at this point is negligible if no more than 5-6 drops are needed). Solid K_3PO_4 is used for the adjustment if the pH should drop below 4.5. The pH paper used for the adjustment is Accutint No. 70.

3. Colorimetry. Colorimetry consists essentially of the addition of CNBr and metol to this decolorized extract. The optimum temperature for the CNBr addition is 75-80° C. and the optimum pH is 4.5. A 10% KH_2PO_4 solution is used to keep the solution at this optimum pH.

The unknown solutions are read against a reagent blank and a niacin standard solution. To correct for any residual color in the decolorized extract, a blank is run for each unknown. The blanks are read against distilled water.

Duplicate colorimetry is performed on each extract by colorizing a 3 ml. and a 5 ml. aliquot (a 3 ml. and a 5 ml. blank must be read for each extract). Besides the obvious advantage of running duplicates at this point for increased accuracy and precision, this procedure also increases the likelihood that at least one of the two aliquots will give a reading within a suitable range of the colorimeter (10-25 γ/tube).

The following detailed scheme of action has been found helpful and is, therefore, presented here.

1. Turn on constant temperature bath set at 75-80° C. and remove CNBr and *ca.* 6 ml. niacin stock standard from refrigerator to allow both to come to room temperature.

2. Assemble and mark enough tubes to run the batch (calibrated at 20 ml. for the blanks and uncalibrated for the unknowns). Two tubes (3 and 5 ml.) are needed for each unknown; two tubes are needed for the standard (3 and 5 ml.) and one each for the reagent blank and for the distilled water blank.

3. Pipette 1.0 ml. of 10% KH_2PO_4 buffer (using 5 ml. Mohr pipette) into each tube (unknowns, blanks, standards, reagent blank, distilled water blank).

4. Add 3 and 5 ml. of each unknown to its respective unknown and blank tubes.

5. Accurately subdilute the niacin stock standard 5:250 in a volumetric flask with distilled water.

6. Add 3 and 5 ml. of the diluted standard (containing 5 γ/ml.) to the respective standard tubes.

7. Add distilled water to all the blank tubes to make up to the 20 ml. calibration; stopper with No. 3 rubber stoppers, mix by inverting each 5 times and put into a separate rack.

8. Pipette enough distilled water into unknown, reagent blank and standard tubes to make up to a total volume of 9 ml. (*e.g.,* 1 ml. buffer and 3 ml. unknown, add 5 ml. H_2O). Stopper all tubes.

9. Place tubes in rack in constant temperature bath at 75-80° C., loosen stoppers and leave undisturbed for 5 min.

10. During that time prepare 5% metol solution. See section on reagents.

11. List all the tubes on a pad and during addition of CNBr note the exact time of addition for each tube so that it may be removed from the constant temperature bath exactly 5 min. after the addition of CNBr.

12. Add CNBr from a 1.00 ml. blow-out pipette to each tube.

13. Five min. after this addition, remove each tube and place it in another rack in a pail of cold water to stop the reaction of CNBr with niacin.

14. When all the tubes have cooled, add 10 ml. metol (clear, color-

less solution) to each tube. Stopper tightly and mix by inverting several times.

15. Place all tubes in the dark for 75 min.

16. Set the colorimeter with filter at 400 mμ to 100 with the distilled water blank and note the setting after the tube is removed. Read all the blank tubes against that setting.

17. Set the colorimeter to 100 with the reagent blank and note the setting after this tube is removed. Read the two standard tubes and all the unknown tubes against that setting.

4. Calculations. The galvanometer readings G are converted to photometric density L according to $L = 2 - \log G$. From the L value of the unknown tube, the L value of the corresponding blank is subtracted, giving the L value due to niacin present ($L_{\text{corr.}}$).

To calculate the calibration constant K, average the following:

$$\frac{L_{3\text{ ml.}}}{15} \text{ and } \frac{L_{5\text{ ml.}}}{25},$$

where $L_{3\text{ ml.}} = L$ value of the standard tube containing 3 ml. of working standard ($= 15\ \gamma$),

$L_{5\text{ ml.}} = L$ value of the standard tube containing 5 ml. of working standard ($= 25\ \gamma$).

The concentration of niacin is then calculated from

$$\frac{L_{\text{corr.}}}{K_{\text{av.}}} \times \frac{1}{1000} \times \text{dilution factor} = \text{mg. of niacin/sample},$$

where $L_{\text{corr.}} = L_{\text{unknown}} - L_{\text{blank}}$

$K_{\text{av.}} =$ average calibration constant.

d. Notes on the Procedure.

1. Some types of samples (*e.g.*, rice, corn grits) require preliminary maceration to permit successful extraction of niacin from the material; blending in a Waring blendor, preceded by an hour's boiling in water or dilute (0.1 N) acid, has been found satisfactory.

2. With some high potency samples, principally pharmaceuticals, decolorization may be omitted. In this case, the hydrolyzate is diluted so that the final dilution contains 3-4 γ/ml.

3. It is advisable to remove as much sediment as possible from the hydrolyzate before adsorption on Lloyd's reagent since failure to do so may result in a more highly colored eluate and a higher blank.

4. Dann and Handler (192) pointed out that up to 200 γ of pure niacin can be taken through adsorption, elution, and lead treatment without measurable loss.

5. The eluate must be made as colorless as possible. The blank should not give an *L* value which is more than 20% of the total *L* value and should preferably be much less.

6. The intensity of the color developed by niacin upon treatment with CNBr and metol depends upon temperature at CNBr addition, amount of CNBr added, time of reaction of CNBr with niacin, pH of extract, *etc.* Conditions should, therefore, be standardized as much as possible to insure that the standard tubes receive exactly the same treatment as the unknown tubes. Small fluctuations in the *K* from batch to batch will take care of small variations in technique, but within any one batch, only vigorous standardization of conditions will assure accurate results. Goldthorpe and Tippit (200) have recently claimed greatly increased sensitivity and reproducibility when (a) the CNBr reaction is carried out at 40° C. for 5 min., or better, at 25° C. for 30 min., (b) the pH is carefully controlled with limits of 4.9-5.3, and (c) the reaction is conducted in the dark.

7. After 75 min. from the time of metol addition, the maximum color has developed. The color remains at the maximum for 15-20 min. and then begins to fade very slowly. The color should therefore be read between 75 and 90 min. after metol addition.

8. Sometimes it is not possible to arrange for a concentration of 4 γ/ml. of final extract. It is then possible to remedy the situation by taking a larger or smaller (8 ml. to 1 ml.) aliquot of the final extract for colorimetry so that the colorimeter tube still contains 10-25 γ of niacin or as much as is possible with 8 ml.

9. One way of assessing the reliability of a method of analysis consists of comparing results with those given by a totally unrelated method. In our laboratory, the average difference between the method described above and the microbiological method of Snell and Wright (201) as modified by Krehl, Strong and Elvehjem (202) was ± 9.4% for 64 random samples. A breakdown according to the types of sample showed that agreement was best on high potency pharmaceuticals (± 3.8%), which require only the abridged chemical method, while various types of low-potency foods showed a mean difference of ± 11.8%.

B. PROCEDURE WITH ANILINE

After Melnick (179)

a. Reagents.

Hydrochloric Acid, (C.P.) concentrated.

Sodium Hydroxide, (50%) equal weights of NaOH (C.P.) and water.

Sodium Hydroxide, 0.5 *N* aqueous solution.

Sulfuric Acid, 0.2 *N* aqueous solution.

Lloyd's Reagent.

Lead Nitrate, anhydrous, powdered.

Phosphoric Acid, 20% aqueous solution.

Potassium Phosphate, K_3PO_4, anhydrous, powdered.

Phenolphthalein, 1% in 95% alcohol.

Methyl violet, 0.1% in water.

Buffer Solution. Composed of 1960 ml. of water, 30 ml. of 15% NaOH, 8 ml. of 85% H_3PO_4, and 333 ml. of absolute ethyl alcohol.

CNBr Solution (4%). Prepared like the 3% solution described above.

Aniline Solution. Redistilled aniline is dissolved in absolute ethyl alcohol to make a 4% solution. When stored in a brown glass bottle at room temperature, this reagent will keep for months.

Standard Niacin Solution. One hundred mg. of U.S.P. Reference Standard are weighed into a 1 l. volumetric flask and made to the mark with absolute ethyl alcohol. This solution will keep indefinitely.

b. Apparatus.

Photoelectric colorimeter with filter at 420 mμ.

Test tubes, calibrated at 15.0 and 16.5 ml.

Test tubes, accurately graduated in 0.1 ml. quantities to 15 ml.

c. Procedure.

A sample containing from 20 to 100 γ of niacin is weighed into a test tube calibrated at 15.0 and 16.5 ml. Ten ml. of water and 5 ml. of conc. HCl are added. The tube is immersed in a boiling water bath and the hydrolysis allowed to proceed for 30-40 min. with occasional stirring. The sample is cooled to room temperature and the pH adjusted to 0.5-1.0 with 50% NaOH, using methyl violet as the external indicator. 2.5 g. of Lloyd's reagent are added and the suspension shaken vigorously for about 1 min. After centrifugation, the supernatant liquid is discarded and the residue washed twice with 10 ml. of 0.2 *N* H_2SO_4, care being taken to disintegrate it completely by shaking vigorously at each washing. The suspensions are centrifuged and the supernatants discarded. To the washed residue 12 ml. of 0.5 *N* NaOH are added and the mixture shaken vigorously for 1 min. Water is then added to bring the volume to the 16.5 ml. mark (the volume of solution is then 15.0 ml.). The tube is again shaken and the resultant suspension centrifuged. The eluate is drained as completely as possible into a clean, dry test tube containing 0.7 g. of finely powdered lead nitrate.

After shaking for 1 min., the suspension is centrifuged and the decolorized solution transferred to another clean, dry test tube. One drop of phenolphthalein is added, followed by solid K_3PO_4 until the color of the solution becomes slightly pink. One to two drops of 20% H_3PO_4 is added from a capillary pipette until the solution is neutral to litmus, the latter being used as an external indicator. The suspension is centrifuged. The clear supernatant solution is drawn by oral suction through a U-tipped pipette into a test tube, accurately graduated in 0.1 ml. quantities to 15 ml.

The volume of the collected supernatant solution is noted and then brought to the 10 ml. mark. For the colorimetric tests 3 ml. portions of the test solutions are pipetted into 3 colorimeter tubes. (a) To the first sample, 7 ml. of the alcoholic buffer solution are added. (b) To the second aliquot, 6 ml. of CNBr reagent are added from a burette, followed in exactly 10 min. with 1 ml. of the aniline reagent. The solutions are stirred after the addition of each reagent. (c) To the third aliquot, 0.1 ml. of the standard nicotinic acid solution is added, followed by the reagents as in (b).

The yellow color developed in solution (b) and (c) and the residual color of solution (a) are read after 5 min. in a photoelectric colorimeter, using a 420 mμ filter. By subtracting from the photometric density of solution (b) that of solution (a) due to the color remaining after decolorization, the photometric density of the reacted niacin is obtained. This is converted into absolute units of niacin by correlating the increment in photometric density, solution (c) − solution (b) with the amount of added niacin.

In the colorimetric measurements it is necessary to have two center settings, one for evaluating the residual color in the extract, the other for the color developed by the chemical reaction. A tube containing 3 ml. of water and 7 ml. of buffer is used to set the colorimeter for the blanks (tubes (a)); a tube containing 3 ml. of water, 6 ml. of CNBr, and 1 ml. of aniline is used to set the instruments for tubes (b) and (c).

Galvanometer readings are converted into photometric density (L) according to

$$L = 2 - \log G$$

(b) − (a) = L_u = photometric density due to unknown.

(c) − (b) = L_i = photometric density due to 10 γ increment.

$\frac{L_u}{L_i} \times 10 \times$ Dilution factor = mg. of niacin in sample.

d. Notes on the procedure.

a) In comparing this method with the microbiological method of Snell and Wright (201), Melnick (179) found that the average difference between the two methods was about 10% for 3 collaborative samples.

b) Shaker and Latif (203) studied the effect of light and temperature on the cyanogen bromide-aniline reaction and recommended that it be carried out at low temperature in order to diminish fading of the color and the occurrence of turbidity. Mueller and Fox (204) found that an ammonia buffer could replace the aniline. These authors reported that the use of buffer together with spectrophotometric measurement of the color gave greater sensitivity and precision, while the effect of varying pH was prevented and less time was required.

C. *p*-AMINOACETOPHENONE PROCEDURE

After James, Norris and Wokes (187)

a. Reagents.

Hydrochloric Acid, concentrated.

Hydrochloric Acid, 6% w/v.

Sodium Hydroxide, 0.1 *N* aqueous solution.

Sulfuric Acid, 0.2 *N* aqueous solution.

Lead Nitrate, anhydrous, very finely powdered.

Phenolphthalein solution.

Thymol Blue solution.

Bromothymol Blue solution.

Tribasic Sodium Phosphate.

Phosphoric Acid, 20%.

Lloyd's Reagent. Each batch should be checked for adsorption and elution of nicotinic acid.

Cyanogen Bromide reagent. Prepare a saturated solution of bromine in distilled water. Cool it below 10° C. and add, drop by drop, an ice cold 10% solution of KCN until it is just decolorized. Prepare this solution fresh each day and keep it in a cool, dark place.

p-Aminoacetophenone, 10% solution in 96% ethanol. If the material exhibits a strong yellow color in acid solution, it should be heated in aqueous solution with adsorbent charcoal and recrystallized from water so that it gives a low reagent blank.

Standard Nicotinic Acid Solutions. Stock. One hundred mg. of nicotinic acid in 50% alcohol to make 100 ml. *Working solutions* (20 and 10 γ/ml.). Dilute the stock solution 1:50 and 1:100 with 25% alcohol.

b. Apparatus. Spekker Photoelectric Absorptiometer, using Chance's No. 7 and H 503 filters before each photocell. A Chance's No. 6 filter is used in checking the response of the photocell. *Centrifuge tubes,* 25 ml. capacity.

c. Procedure.

1. Preparation of Extract. Mix 1 g. of sample (in No. 30 or 40 powder for barley or malt, and containing up to 150 γ of nicotinic acid) with 5 ml. of concentrated HCl and water to make 15 ml. and place in a boiling water bath for 40 min. Cool to room temperature and centrifuge (A). Decant the supernatant liquid, add to it 2.5 g. of Lloyd's reagent, shake for 1 min. and centrifuge (B). Reject the supernatant liquid B. Wash the residue from A with 10 ml. of 0.2 *N* H_2SO_4 and centrifuge. Add the supernatant liquid from this to the residue from B. Shake this mixture for 1 min., centrifuge and reject the supernatant liquid. Add to the residue 10 ml. of 0.1 *N* NaOH (more if necessary) to make alkaline to phenolphthalein. Shake vigorously for 1 min. and make up to 16.5 ml. (= 15 ml. of original acid extract). Foam can be dispersed by a drop of alcohol before adjusting to volume. Centrifuge, decant and reject the residue. To the supernatant liquid add very finely powdered $Pb(NO_3)_2$ (Anala R) to bring the pH to between 8.0 and 9.0, using thymol blue as external indicator and keeping the liquid well stirred to avoid adding an excess. Centrifuge and add to the supernatant liquid one drop of phenolphthalein indicator. If a pink color is not obtained, add sufficient solid Na_3PO_4 to produce a pink color and centrifuge again. Take 5 ml. of the supernatant liquid and add 1 drop of 20% H_3PO_4 and then Na_3PO_4 to bring the pH to 6.5 ± 0.3, using bromothymol blue as external indicator. Make up to 25 ml. (= "final extract").

2. Development of Color. Four mixtures of the test solution are prepared as folows.

a) Duplicates containing 5 ml. of test extract plus 1 ml. of water; b) 5 ml. of test extract plus 1 ml. of nicotinic acid solution (10 γ/ml.); c) 5 ml. of test extract plus 1 ml. of nicotinic acid solution (20 γ/ml.). A total reagent blank is set up containing 6 ml. of water. To each of these 5 tubes add 2 ml. of CNBr reagent, heat in a water bath at 56-58° C. for 4 min., cool to 20° C. for 5 min., and then add to each 1 ml. of *p*-aminoacetophenone solution and 1 ml. of 6% HCl. Keep the mixtures in the dark and 5 min. after adding the *p*-aminoacetophenone and HCl measure the optical density of a, b and c against the total reagent blank.

Prepare an acid alcohol blank by mixing 20 ml. of alcohol, 20 ml. of 6% HCl, 14.9 ml. of 0.1 *N* NaOH and water to make up 100 ml.

Prepare a solution blank by mixing 4 ml. of final extract with 4 ml. of the acid alcohol blank. Measure its optical density (d) against water mixed with an equal volume of the acid alcohol blank.

3. Calculation.

$$\frac{a-d}{b-a} \times \frac{15}{1} \times \frac{25}{5} \times \frac{10}{5} = \gamma/\text{g}.$$

or

$$\frac{a-d}{c-a} \times \frac{15}{1} \times \frac{25}{5} \times \frac{20}{5} = \gamma/\text{g}.$$

d. Notes on the procedure.

1. Fairly complete recoveries were obtained of nicotinic acid added after decolorizing or before extraction of the foodstuff.

2. This method has given satisfactory reproducibility and agreement with microbiological results on a wide range of cereal products. This method gave considerably higher results than the microbiological method on low-extraction flours, maize, and some yeast preparations.

VII. Pyridoxine

1. HISTORICAL DEVELOPMENT

Following the recognition of pyridoxine as a distinct member of the B complex by György and his associates (205-210), and its isolation and identification by two groups of investigators (211-215; 216-219), various colorimetric methods of assay were proposed. The reaction of phenols with the Folin-Denis reagent (220, 221) was mentioned by some investigators (222, 223), but did not find general acceptance due to its lack of specificity. Similarly, the reactions of pyridoxine with diazotized sulfanilic acid (223, 224) or diazotized *p*-aminoacetophenone (225) have not been widely endorsed. Other less common assay methods involve the formation of cyanine dye (222) and the reaction of pyridoxine with $FeCl_3$.(212, 226).

The most widely used colorimetric reaction for pyridoxine is based on the indophenol test developed by Gibbs (227). Stiller, Keresztesy and Stevens (213) observed that pyridoxine gave a positive Gibbs test; Scudi and coworkers (228-231) developed a method of analysis based on the reaction of pyridoxine with 2,6-dichloroquinonechloroimide. Since this dye couples with many other substances, Scudi proposed the use of a 2-phase system and introduced the principle of a borate blank; this blank is run by coupling pyridoxine with a buffered solution of boric acid prior to colorimetry, thus preventing color formation of the dye with the vitamin. However, because it is not specific for pyridoxine, the borate blank could be used only to recognize interferences which must then be removed by other treatments. Bird, Vandenbelt and Emmett (232) suggested that pyridoxine be removed from interfering substances by adsorption on superfiltrol prior to colorimetry. Hochberg, Melnick and Oser (233-235), while retaining the color reagent and the principle of the borate blank, developed a method based on the coupling of the dye with pyridoxine in a one-phase system. The use of a strong NH_4OH-NH_4Cl buffer of high basicity was found to reduce interference due to the different kinds and amounts of bases and salts in the test solutions.

Under the conditions established in this procedure, the borate blank provides a quantitative correction for most other substances which can couple with the dye, so that colorimetry is more specific for pyridoxine. For biological materials, adsorption on Lloyd's reagent was included to isolate pyridoxine.

While this developmental work was in progress, it was found that, besides free pyridoxine, a conjugated form of the vitamin is present in rice bran concentrates (236) and other materials (237). Acid hydrolysis prior to the colorimetric procedure was proposed to liberate free pyridoxine from the bound form.

Elucidation of the nature of the B_6 group has placed serious doubt upon the applicability of any chemical method to the determination of the vitamin in natural materials. Following the discovery of "pseudopyridoxine," a physiologically active metabolite of pyridoxine in natural products (238), Snell and associates (239) in conjunction with Harris and coworkers (243) established the presence in natural materials, the biological activity, and the structure and synthesis of two other members of the vitamin B_6 group, pyridoxal and pyridoxamine. Pyridoxal and pyridoxamine are present in varying amounts in most natural products and often constitute a considerable percentage of the vitamin B_6 content (240, 244). Melnick and coworkers (245) noted that, by their colorimetric method (234), pyridoxamine is about 31% as reactive as pyridoxine on an equimolar basis, and pyridoxal only 16%. They pointed out that the procedure may be adapted to the specific measurement of pyridoxine in pure systems containing varying proportions of pyridoxal and pyridoxamine, but that considerable caution must be exercised in applying this method to any material in which pyridoxine does not constitute a major portion of the vitamin B_6 content. Ormsby, Fisher and Schlenk (246) found it possible to distinguish between pyridoxal, pyridoxamine and pyridoxine by the difference in color obtained on treatment with diazotized sulfanilic acid. By determination of the absorption spectra of the dye, these authors were able to make a qualitative and an approximate quantitative estimate of the B_6 component of glutamic-aspartic transaminase. In its present status, however, the chemical method is not recommended for the assay of most natural materials. Its use should be confined to analytical samples, such as pharmaceutical preparations, which are known to contain pyridoxine as the only member of the B_6 group.

2. METHOD OF ANALYSIS

The procedure presented here is essentially that of Hochberg, Melnick and Oser (233, 234), based upon the work of Scudi *et al.* (230, 231).

a. Reagents.

Chloroimide Reagent. One hundred mg. of recrystallized 2,6-dichloroquinonechloroimide are dissolved in 250 ml. of isopropanol. The solution is stored in a glass-stoppered bottle in the refrigerator and portions are withdrawn as needed. The reagent should not be kept for more than one month and should be discarded sooner if a pink coloration develops. To recrystallize the solid, about 1 g. is dissolved in 50 ml. of acetone in a 400 ml. beaker and, gradually, about 200 ml. of water are added with continuous stirring. The crystals are collected on Whatman No. 42 filter paper in a small Buchner funnel, rapidly air-dried by suction, and stored in a sealed bottle in the refrigerator. The recrystallized reagent is stable for more than 6 months under such conditions of storage.

Ammonia-Ammonium Chloride Solution. 160 g. of NH_4Cl, C.P., are dissolved in 700 ml. of water and 160 ml. of concentrated ammonia water (approximately 27%) added. The solution is diluted with water to 1 l.

Boric Acid Solution. Five g. of powdered boric acid, C.P., are dissolved in 100 ml. of distilled water.

Pyridoxine Hydrochloride Standard. One hundred mg. of U.S.P. Pyridoxine Reference Standard are dissolved in 1000 ml. of 0.1 *N* HCl. This stock solution is stable for at least 3 months if stored in the refrigerator in an amber bottle. Working standards are prepared daily by dilution of the stock solution.

Sulfuric Acid. 5.0 *N*.

Sodium Hydroxide. 2.0 *N*.

Manganese Dioxide. C.P.

Isopropanol. C.P. 99%, Baker's.

2. Apparatus.

Photoelectric Colorimeter. Preferably direct-reading with filter at 620 mμ.

3. Procedure.

The method of analysis is here applied to a multivitamin tablet, but may easily be adapted to any other sample known to contain pyridoxine as the only member of the vitamin B_6 complex. The final aliquot for colorimetry should contain no more than 20 γ of pyridoxine.

Enough tablets to give 2-6 mg. of pyridoxine are placed in a blendor, 100 ml. of distilled water and 8.3 ml. of 5 *N* H_2SO_4 (note a) are added; the mixture is blended for 2 min., a few drops of caprylic alcohol are added and the solution transferred quantitatively to a 250 ml. red volumetric flask (total volume should not exceed 200 ml. at this point). The mixture is heated for 20 min. on the steam bath, cooled, made up to volume, and mixed thoroughly. Fifteen ml. of this solution are pipetted into a 50 ml. glass-stoppered centrifuge tube, 5.0 ml. of 2 *N* NaOH and about 200 mg. of MnO_2 are added (note b), the mixture is shaken for 5 min. and centrifuged lightly. Five ml of supernatant and 25 ml. of isopropanol are pipetted into a 50 ml. glass-stoppered centrifuge tube, the mixture is shaken vigorously and centrifuged at 2500 r.p.m. for several minutes.

Colorimetry.

To 6.0 ml. of extract are added 2 ml. of NH_4OH-NH_4Cl buffer, 1 ml. of H_3BO_3 solution and 1 ml. of chloroimide reagent and the colorimeter is set at 100 (620 mμ filter) exactly 1 min. 20 sec. after the first drop of the chloroimide reagent has been added from a rapid delivery pipet. An equal aliquot of extract is treated in exactly the same way, except that water is added in place of the H_3BO_3 solution.

A standard is run in duplicate with each batch of unknowns: 10.0 ml. of stock standard are diluted to 100 ml. with water; 1.0 ml. of this dilution + 5.0 ml. of isopropanol are treated for colorimetry exactly as the 6.0 ml. aliquot of unknown extract. The following table summarizes the steps of colorimetry for unknowns, standards, and internal standards.

	Unknown Extracts 5.0 ml. + 25 ml. isopropanol, mix, then	Blanks 5.0 ml. + 25 ml. isopropanol, then	Standards	Unknowns with Increment (note c) 2.0 ml. + 3.0 ml. water + 25 ml. isopropanol, then
Aliquot of extract	6.0 ml.	6.0 ml.	1.0 ml.	6.0 ml.
Isopropanol	—	—	5.0 ml.	—
Ammonia buffer	2.0 ml.	2.0 ml.	2.0 ml.	2.0 ml.
Boric acid solution	—	1.0 ml.	—	—
Water	1.0 ml.	—	1.0 ml.	—
Increment solution	—	—	—	1.0 ml.
Chloroimide reagent	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.

Calculations.

Convert all galvanometer readings (G) to photometric densities (L) according to

$$L = 2 - \log G.$$

The calibration constant K is obtained from

$$K = \frac{L_s}{C_s}$$

where L_s = photometric density of standard solution
C_s = concentration of pyridoxine in standard solution.

Then

$$\frac{L}{K} \times \frac{1}{1000} \times \text{dilution factor} = \text{mg. of pyridoxine/sample.}$$

4. Notes on the Procedure.

a) Acid extraction has been found to yield results for multivitamin preparations which are somewhat higher and in better agreement with microbiological assays by *S. carlsbergensis,* than extraction with 0.5 *N* NaOH, as originally proposed by Hochberg *et al.*

b) MnO_2 eliminates interference by ascorbic acid.

c) An internal standard may be run if masking of the color is suspected. Fifteen ml. of stock standard are diluted to 200 ml. with water (this increment solution contains 7.5 γ/ml.). The unknown extract is subdiluted in the following way: in the last step before colorimetry, instead of shaking 5.0 ml. of supernatant with 25 ml. of isopropanol, 2.0 ml. of supernatant + 3.0 ml. of water are shaken with 25 ml. of isopropanol. Six ml. of this extract (containing 7.5-10 γ of pyridoxine) are colorized as usual and in addition an increment tube is set up in which 1.0 ml. of the increment solution is added in place of 1.0 ml. of water (see table).

d) The calibration curve is linear up to 20 γ of pyridoxine per tube.

e) Both the calibration constant and the time curve must be checked whenever a new lot of chloroimide reagent, buffer or isopropanol is used.

VIII. Choline

There are several chemical methods which have been recommended for the determination of choline. In food products and other biological material, choline is present chiefly in the form of phospholipides or acetylcholine. For instance, in blood plasma it is estimated that less than 5% of choline is in the free form (247). For the estimation of total choline values preliminary hydrolysis is indispensable.

The old platinum chloride method (248) and the gravimetric reineckate method of Kapfhammer and Bischoff (249) are not easily adaptable for general use. The colorimetric modification of the method of Kapfhammer and Bischoff, by Beattie (250) and later by others, and the colorimetric periodide methods of Sharpe (251) and Roman (252), with several modifications, are at present the preferred chemical procedures for the quantitative determination of choline.

1. Colorimetric Reineckate Method

As first proposed, the method of Beattie (250) consisted in the precipitation of choline with a freshly prepared saturated solution of ammonium reineckate. The precipitate was dissolved in acetone and the reddish color imparted to the acetone was compared with that of a standard solution containing a known amount of choline reineckate in acetone, or with a specially prepared methyl red solution of the same color. This method proved to be unsatisfactory because of the difficulty of making accurate measurements with a visual colorimeter (253). Furthermore, only rather concentrated solutions furnish sufficient color for accurate determination by this method.

Reagents.

Ammonium Reineckate.

$NH_4[Cr(NH_2)_2(SCN)_4].H_2O$ may be prepared according to the method of Dakin (254) or purchased as the pure salt from biological supply houses. It is stored at low temperature.

Acetone.

Methanol.

n-Propanol.

Barium Hydroxide. Saturated 9 or 10% solution (247) 10 g. of $Ba(OH)_2$ dissolved in 100 cc. of hot distilled water and filtered.

Glacial Acetic Acid.

Thymolphthalein Indicator.

Choline Chloride. For standardization, solutions of known choline content are prepared. A quantity of choline chloride, which is very hygroscopic, is recrystallized 4 times from absolute ethanol, introduced into a weighed bulb, and dried at 84° C. *in vacuo* for 48 hr., after which the bulb is sealed and weighed. The sample is then dissolved in water and adjusted to volume.

The following method is based chiefly on the recommendation of Jacobi, Baumann and Meek (255), Engel (256), and Glick (257).

The weighed sample, if necessary minced or ground finely, containing the equivalent of 2-5 mg. of choline chloride, is placed in an alundun thimble of medium porosity (80 mm. long × 22 mm. in diameter) for extraction in a Soxhlet apparatus fitted with a 125 ml. boiling flask. About 100 ml. of methanol are used as the solvent and a boiling chip is added. The extraction is allowed to proceed for 24 hr. on an electric hotplate. With some finely divided materials such as flour, the tendency to form a hard cake makes it desirable to mix the sample intimately with No. 2 pulverized pumice to facilitate the extraction. Plasma (and presumably other liquid samples) may be extracted directly with 30 volumes of methanol for 1 hr. at 55° C.

The boiling flask containing the methanol extract is placed on a steam bath and, when only a few ml. of solvent remain, 30 ml. of a saturated (or 10%) solution of $Ba(OH)_2$ are added, and the heating is continued for 90 min. After the mixture is cooled, a drop of 1% alcoholic thymolphthalein is added to the hydrolyzate, and glacial acetic acid is introduced until the blue color just disappears. Thymolphthalein is to be recommended instead of phenolphthalein (255) for the neutralization, as the color does not fade and the color change is easier to detect. The mixture is then filtered by suction through a sintered glass filter tube of medium porosity (15 to 30 ml. capacity) into a 125 ml. suction flask. The boiling flask is rinsed with small portions of distilled water and the rinsings are used to wash the filter, a total of about 15 ml. of water being used. To the combined filtrate and washings, 6 ml. of a 2% solution of reinecke salt in methanol are added, and the flask is placed in a refrigerator at about 5° C. for 2 hr. or longer, up to 12 hr. (258).

The bright red choline reineckate precipitate is separated by filtering with suction through a 30 ml. sintered glass filter tube of medium porosity. When the precipitate is dry, it is washed 3 times with 2.5 ml. portions of *n*-propanol, and again dried by means of the suction. A tube graduated at 10 ml. (14.5 cm. long × 1 cm. diam.) is placed inside the 500 ml. suction flask so that the stem of the filter tube can be inserted into its mouth. The choline reineckate on the filter is dissolved in a few ml. of acetone and, by means of gentle suction, the solution is drawn into the 10 ml. tube. After the pink solution is mixed, it is used for photoelectric determination, with a filter which transmits light at about 520 mμ.

The final result, expressed in mg. of choline chloride, is obtained by reference to a calibration curve constructed from the color intensities given by solutions of known choline chloride concentration. The absorption of light was found to be a linear function of concentration over the range of 0.5-9 mg. Thus, the method does not yield reproducible results for amounts of choline less than 0.5 mg.

The choline reineckate is perfectly stable in acetone for at least 30 min. (253). This is a much longer time than that actually necessary for the measurement of the transmittancy of a sample.

Replacing acetone, which is a volatile solvent, with 60% acetone in water yields a less volatile solvent which still instantly dissolves choline reineckate (256). This recommendation has not yet been confirmed.

Compounds, other than choline, that form insoluble reineckates exist in many biological materials or can be liberated from them on hydrolysis.

Interference by such substances is eliminated to a considerable degree by precipitating the reineckate in an alkaline medium as insured in the above method by the use of thymolphthalein as indicator.

A further modification of the original reineckate method was proposed by Marenzi and Cardini (259). It is claimed—without confirmation—that this new modification allows choline to be determined in samples containing as little as 15 mg. It is based on the oxidation of the chromium of choline reineckate to the chromic state by means of alkaline H_2O_2 (perhydrol), followed by colorimetric determination of chromate by means of the color produced in acidic solution with a solution of diphenylcarbazide (Cazeneuve's reaction).

The amount of choline to be determined varies between 75 and 100 mg. Precipitation with ammonium reineckate in the samples which may be prepared according to the reineckate method described above should take place in a centrifuge tube with a slender end (± 3 mm.). The tube is placed in ice water for at least 20 min. to complete the precipitation of choline. The precipitate is separated by centrifugation for 4 min. at 3,000 r.p.m. At the end of the period of centrifugation, the supernatant is removed by means of a small capillary tube with a fine end and provided with a suction bulb. The precipitate is then washed 2 or 3 times with 0.5-1.0 ml. of ethanol (95%) or preferably with *n*-propanol.

The choline reineckate thus obtained is dissolved in about 1 ml. of acetone, warmed if necessary, and the solution is transferred to an ordinary test tube. The centrifuge tube is then washed 2 or 3 times with 1 ml. of 60% acetone, and the washings are transferred to the same test tube. One to 2 ml. of water, 0.2 ml. of NaOH (10%), and 1.1 ml. of perhydrol for each 50 γ of choline in the sample are added. An excess of perhydrol does not interfere, but unnecessarily prolongs the period of heating in order to destroy the excess.

The test tube thus prepared is placed in a boiling water bath. After most of the acetone is evaporated, the tubes are kept in the bath for 20-30 min., being shaken frequently in order to accelerate the decomposition of the perhydrol which must be completely destroyed, as otherwise no color will form. The final heating is conveniently carried out over a flame for a few seconds to insure complete destruction of the perhydrol.

During heating, the liquid acquires a yellow color from the formation of chromate: occasionally the initial color due to the chromate is deepened by a color produced by other substances, an indication of insufficient perhydrol. In this case, 0.1 ml. more of perhydrol is added and the heating is continued.

After the oxidation of the chromium, the tubes are cooled, diluted with 3-4 ml. of water, 2 ml. of H_2SO_4 (10% by volume) are added, and sufficient diphenylcarbazide solution (0.2% in 96% alcohol) is added to yield a final concentration of 8%. A violet-red color appears. The liquid is diluted to the appropriate volume and used for spectrophotometric or photoelectric determination, with a filter which permits transmission of light at about 520 mμ.

As in the original reineckate method, various dilutions of choline solutions serve as reference for calculating the amount of choline in the unknown samples analyzed.

2. PERIODIDE (ENNEAIODIDE) METHOD

The principle is to extract the choline from the blood, tissues, and various other products, and to precipitate it with iodine as periodide, to wash off any free iodine and to decompose the periodide with dilute HNO_3, finally to extract the free iodine with chloroform and estimate it using a standard solution of $Na_2S_2O_3$ (251, 252, 260). The original method of Sharpe required rather large amounts of choline, corresponding to 20-50 ml. of blood. By using aqueous solutions of choline chloride, Roman obtained reproducible results within a range of 5 γ-5 mg. of choline (± 5%). In comparative studies (253), Roman's method yielded consistently lower values (by about 10%) than the colorimetric reineckate method. The greatest source of error of the periodide method is the volatile and unstable nature of the choline periodide precipitate. Furthermore, potassium periodide is not a specific precipitant for choline, but yields precipitates with betaines, dimethylamine, trimethylamine, certain cyclic bases, and with many alkaloids (261). Thus, in the presence of such compounds, for instance, in plant extracts, it is impossible to relate the titration figures obtained by the periodide method to definite chemical entities. It is claimed (261) that purification of plant extracts by means of lead acetate and Norit removes the greatest part if not all of the periodide precipitable compounds with the exception of choline.

The following procedure has been found to be suitable by Reifer (261): An extract of 5 g. of dry tissue in 250 ml. of water is made up in the usual way, and the extract cleared by centrifuging. Five ml. of a saturated solution of neutral lead acetate are added with shaking, the suspension centrifuged, and lead removed from the supernatant liquid by means of H_2S. After removing PbS, 200 ml. of the cleared solution (corresponding to 4 g. of tissue) are evaporated *in vacuo* to small volume and made to 25 ml. The solution is heated for 3 min. in a boiling water bath with 0.5 g. Norit, and the charcoal removed by filtration. On a 1 ml. aliquot the exact amount of 0.2 *N* NaOH required to bring the solution to pH 7 is determined. For the estimation of choline an aliquot of

10 ml. is taken, the predetermined quantity of NaOH is added, and the volume made up to 12 ml. For the estimation, an aliquot of 3 ml. equivalent to 0.4 g. of tissue is taken. The analysis is carried out as follows:

Reifer's Modification of the Periodide Method (261).

Reagents. Potassium Periodide Solution. Dissolve 2.5 g. iodine and 3.75 g. KI in a little water and make to 100 ml., sufficient NaCl being added to make the final solution 10%. The solution should be made up 24 hr. before use, and keeps for at least a month.

Aluminum Hydroxide Suspension. Dissolve 10 g. potassium alum in 150 ml. water and add 100 ml. 0.2 *N* NaOH.

Sodium Chloride. 10% solution.

Sulfuric Acid. 5% solution.

The above solutions are all used at low temperatures and should be stored in the refrigerator.

Sodium thiosulfate. 0.01 *N* and 0.02 *N*.

Starch solution. 0.25% solution.

Ethanol. 90%.

Three ml. of a neutral solution containing between 0.1 mg. and 5.0 mg. of choline are brought into a 25 ml. centrifuge tube which is kept in ice water. After the temperature of the contents of the tube has dropped below 10° C., 5 ml. of periodide solution are run in slowly, with shaking, and the tube put in the refrigerator. Crystallization of the precipitate is complete after 3 hr., when the tube is shaken and centrifuged for 2 min. Five-tenths ml. of $Al(OH)_3$ suspension is then carefully added so that it forms a continuous layer on top of the liquid. The tube is centrifuged for 5 min. at 2500 r.p.m. and the supernatant liquid is sucked off without disturbing the $Al(OH)_3$ layer. The walls are twice washed down with 3 ml. of ice cold 10% NaCl solution, which is sucked off without centrifuging.

To remove $Al(OH)_3$, 2 ml. of cold 5% H_2SO_4 are run in, the hydroxide layer gently agitated with a thin glass rod without disturbing the periodide precipitate, and the material centrifuged for 3 min. Because of the volatility of iodine it is essential to carry the estimation to completion as rapidly as possible. The supernatant liquid is sucked off and the precipitate dissolved, with stirring, in 1 ml. of 90% alcohol. After the addition of 5 ml. of water the solution is titrated with thiosulfate in the usual way.

Erickson and her collaborators (262) presented further modifications of the choline periodide method of Roman. These modifications include the use of immersion filter sticks which facilitate washing the labile choline enneaiodide precipitate with minimum disturbance at the temperature of melting ice, and the incorporation of a technique for the conversion of iodide to iodate with bromine preceding titration with standard $Na_2S_2O_3$. The latter modification offers the advantage of ready solubility of the precipitate in the bromine solution and 6-fold increase in the final titration value.

Complete recovery can be obtained with samples equivalent to 0.15 mg. or more of choline.

The modification of the choline periodide method proposed by Erickson et al. may be carried out in solutions of choline, in aqueous extracts, prepared without or with preceding hydrolysis of phospholipides present in the samples to be analyzed. Treatment with $Ba(OH)_2$ may follow the same procedure as described above for the reineckate method.

Apparatus. Titer tubes, 1 × 4 in., conical tipped centrifuge tubes, 60 cc. capacity, alundum immersion filter sticks with Filter RA 225 (Scientific Glass Apparatus Co.).

Solutions. Sodium acetate, 25%; formic acid, 90%; potassium iodide, 10% (freshly prepared); starch, 1%; iodine-potassium iodide reagent: 15.7 g. of iodine and 20 g. of KI in 100 cc.; bromine solution: 10% potassium acetate in glacial acetic acid plus 6 drops of bromine/100 cc.; standard $Na_2S_2O_3$, 0.02 *N*, from which 0.005 *N* solution is freshly prepared by dilution.

The centrifuge tubes containing the hydrolyzates are chilled in an ice-salt bath to temperature of 5-10° C. before the addition of 0.4 cc. of iodine-KI reagent for each cc. of solution. After addition of the reagent, the tubes are allowed to stand in the ice bath for 30 min., then centrifuged at high speed for 15 min. and replaced in the ice-salt bath. The supernatant fluid and wash solutions are removed with alundum immersion filter sticks, which enables washing the labile precipitate with minimum losses from volatilization. The precipitate is washed with 1 cc. portions of water (5-10° C.) until the wash solution is colorless (usually 5 washings suffice), with caution to avoid disturbing or sucking the precipitate onto the filter. The filter stick is allowed to remain in the tube while 5 cc. of the bromine solution are added. It is advisable to stir up the precipitate with the filter and then allow the tubes to stand for several hours for complete conversion of the iodide to iodate. The filter sticks are then washed (water being forced through the filter to rinse the inside) and removed.

Just before titration, 2 cc. of a 25% sodium acetate solution and 10 drops of formic acid are added. This should decolorize the solution completely. (Should a yellow color persist, indicating incomplete oxidation of the iodine, more bromine solution should be added and the solution allowed to stand again). Five cc. of a freshly prepared 10% KI solution are added, followed by immediate titration with 0.005 *N* $Na_2S_2O_3$, with 1 cc. of a 1% starch solution as indicator. A blank determination on acidified water in place of the hydrolyzate solution should be made simultaneously and subtracted from the titration values. Calculation is based on the equation that 1 cc. of 0.005 *N* $Na_2S_2O_3$ is equivalent to 0.01122 mg. of choline.

Lintzell and Fomin introduced a new chemical principle (263) for the determination of lecithin in organs (264) and in plasma or total blood (265). It is based on the fact that choline in alkaline solution may be oxidized with permanganate to trimethylamine according to the following equation:

$$OH(CH_3)_3N.CH_2.CH_2.OH + O_2 \xrightarrow{\text{alk. perm.}} (CH_3)_3N + CH_2OH.COOH.$$

Using pure choline chloride for analysis, the trimethylamine may be distilled over into dilute (*N*/50) H_2SO_4 and titrated with *N*/50 trimethylamine against phenolphthalein as indicator. In organs and plasma or blood, choline is not the only N-containing compound which will react with alkaline permanganate solution. As the result of such reaction, ammonia, methylamine, or dimethylamine may be produced, in addition to trimethylamine, the latter being the specific oxidation product of choline. Separation of trimethylamine from these other basic and equally volatile oxidation products may be achieved by the addition of formaldehyde which according to Delepine (266) forms non-volatile compounds with ammonia, mono- or dimethylamine, but does not react with trimethylamine which in consequence remains volatile and may be quantitatively obtained by means of distillation as already indicated.

This interesting principle has been applied originally to the determination of choline in lecithin. It is a time-consuming procedure, the distinct value of which has not yet been proven for the analysis of total (free and bound) choline. The conversion of choline into trimethylamine as basis for quantitative determination of choline has also been utilized by Klein and Linser (267) in their studies on the choline metabolism in plants. The conversion was accomplished by Klein and Linser by heating the choline-containing solution at high temperature (180° C.) in the presence of 33% NaOH. The total volatile bases formed were determined in an aliquot portion of the distillate by the usual procedure. In a second aliquot of the distillate the amount of ammonia present was measured by nesslerization and then deducted from the total figures. The sensitivity of the method is restricted. It requires not less than 20 mg. of choline in the samples to be analyzed.

Neither the method of Lintzel and Fomin, nor the cruder modification of Klein and Linser, appears to be suited in their present form for general use.

A further, less well-known method for the determination of choline is that of Ambo and Aoki (268). It is based on the complete precipitation of choline by alcoholic saturated $HgCl_2$ solution, 0.005 mg. choline binds 0.067 mg. $HgCl_2$.

According to original instructions (268), *ca.* 500 mg. of blood or tissue are left in *ca.* 8 ml. of absolute ethanol for 24 hr. The filtrate is acidified with a few drops of dil. HCl-alcohol and evaporated to dryness. The residue is extracted 3 times with petrol ether, followed by extraction (repeated 3 times) with small amounts of cold absolute ethanol. The combined cold alcoholic extract is filtered through a small filter, the filtrate reduced *in vacuo* to 0.1-0.2 ml. and then mixed with 2 ml. of saturated alcoholic $HgCl_2$ solution. The mixture is left for 24 hr. at cold temperature. After centrifugation (for 10 min. at 2,800 r.p.m.), the supernatant fluid is removed, the residue washed with ether, and finally dissolved in 1 ml. 10% KI solution. One ml. 15% KOH and a few drops of Mucilage gummi arabici and 1 ml. of 11.5% formaldehyde are added. This is followed by the appearance of liberated mercury in fine dispersion. The mixture is transferred into a flask and acidified with 6 ml. of 20% glacial acetic acid. Two ml. of $N/200$ KIO_3 are added, and the excess of iodate titrated with $N/200$ $Na_2S_2O_3$ (with 1% starch as indicator). One-tenth ml. of $N/200$ KIO_3 corresponds to 0.067 mg. $HgCl_2$.

It is improbable that this method, with its original recommendation of cold alcoholic extraction, could furnish reliable analytical data for total choline content (free and bound). Extraction and hydrolysis of the sample in a manner described above for the reineckate method might improve on the technic for which, even in its original form (267), confirmation is still lacking.

IX. Inositol

Chemical methods used in the past for the estimation of inositol in biological material (269-273) were tedious, lengthy, and involved the use of relatively large amounts of material. The recent method of Platt and Glock (274) is much quicker and possesses several other advantages, including improvements in the extraction of inositol from the assay material, the removal of interfering substances by ion exchange procedure and the estimation of inositol, without isolation, by oxidation with periodic acid.

PRINCIPLE OF THE METHOD OF ESTIMATION BY PLATT AND GLOCK (274)

Test-material is extracted with water, and the fractions insoluble in 70% acetone and soluble in ether both removed from the aqueous extract. Glucose is then removed by yeast fermentation, and both acidic and basic substances present in the extract removed by adsorption on a mixture of ion exchange adsorbents. The free inositol present in the extract is then quantitatively oxidized with HIO_4, the excess of HIO_4 being estimated iodometrically. Suitable corrections are applied for any HIO_4 used in oxidizing the glycerol which may be present in the extract at this stage. Water-soluble combined inositol is determined after acid hydrolysis of the aqueous extract.

The method has been applied to animal tissues. Its use for materials from other sources remains to be demonstrated.

TECHNIQUE

Reagents.

Inositol.

Acetone.

Ethyl Ether.

Baker's Yeast.

Zeolite (for cation exchange).[6]

M.P.D. Resin (for anion exchange).[6]

2 N HCl.

2 N NH_4OH.

0.01 M HIO_4.

Phosphate Buffer (12 g. $Na_2HPO_4.12H_2O$ and 20 ml. N H_2SO_4 in 100 ml. water).

5% KI.

0.004 N $Na_2S_2O_3$.

1% Soluble Starch (in saturated NaCl solution).

$NaHCO_3$, Saturated Solution.

Preparation of Tissue for Extraction. As soon as possible after death, the tissue is removed from the animal, sectioned on a freezing microtome, and the sections dried in their layers in Petri dishes *in vacuo* in the cold room over silica gel. Drying is completed in an oven at 110° C., after which the material is powdered and kept in the cold until analyzed. The water content of another portion of the same tissue is determined by drying to constant weight at 110° C.

Extraction of Inositol. An amount of dried powdered tissue, containing approximately 0.5-1.0 mg. inositol (for instance 1.0-2.0 g. dry skeletal muscle powder), is brought to the boil with 50 ml. distilled water, filtered under reduced pressure through a small plug of cotton-wool in a funnel, and the vessel and filter washed with two portions of 5 ml. of distilled water. This process is repeated twice, the 3 extracts pooled and evaporated to about 50 ml., and acetone added to give a final concentration of 70%. The precipitate is filtered off and the acetone removed from the filtrate by distillation. The extract is evaporated down to about 25 ml. and extracted with ether. The ether extract is washed once with about 20 ml. distilled water, and the washings added to the aqueous residue, which is then concentrated to approximately 30 ml. If it is desired to estimate the "combined" water-soluble inositol as well as the "free" inositol, the aqueous extract can be divided into two portions and one hydrolyzed as described below.

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[6] Obtainable from Permutit Co.

Removal of Glucose by Yeast Fermentation. A washed yeast suspension is prepared by shaking one part by weight of pressed baker's yeast with 5 parts of distilled water, and centrifuging; this process is repeated 4 times and the suspension of washed yeast then made up to the original volume with distilled water. The suspension is prepared fresh each day and its potency tested by incubating 2 ml. with 25 ml. glucose solution (containing 5 mg. glucose) for 10 min. at 37° C., and estimating the reducing power of the supernatant fluid, obtained by centrifuging, by one of the sensitive micro methods used for glucose determination, such as Hagedorn and Jensen's ferricyanide method. A blank is set up at the same time with yeast suspension and water only.

The tissue extract is incubated at 37° C. with 2 ml. of the yeast suspension for 10 min. and then centrifuged. The supernatant liquid is siphoned into a boiling tube and the yeast washed twice by centrifuging with 10 ml. portions of distilled water, the washing being kept separate from the main supernatant fluid for the subsequent adsorption.

Preparation of ion exchange materials and the removal of interfering substances from tissue extracts. After treatment with yeast, the extract is purified by the use of ion exchange materials, with a carbonaceous zeolite for cation exchanges and "M.P.D. Resin" as an acid adsorbent. Treatment of the extract with a mixture of regenerated, washed and dried carbonaceous zeolite and "M.P.D. Resin" is more efficient than successive treatments with the two materials separately. With this technique, however, the adsorbing materials cannot be regenerated.

Two hundred fifty g. or more of the carbonaceous zeolite are shaken gently at intervals for a period of 1 hr. with 2 vols. of 2 N HCl, filtered on a Buchner funnel and washed on the funnel with a total volume of approximately 5 l. of distilled water. The final washings should be neutral to litmus. This regenerated, washed cation exchange material is dried at 100° C. and sieved so that the particles are of 20/40 mesh sieve size. The "M.P.D. Resin" is treated in a similar way, but with 2 *N* NH_4OH instead of HCl. At least 1 l. of hot distilled water should be used to wash this reagent, in order to remove excess *m*-phenylenediamine which is liable to be present and which, if not removed completely, gives a blank reading with HIO_4. These 2 regenerated, washed, dried, and sieved materials are mixed in equal parts by weight; this preparation will be called the "adsorbent."

Fifteen g. of adsorbent are introduced into a tube approximately 20 × 7/16 in., narrowed at the bottom to approximately 3/16 in., and fitted with a small piece of rubber tubing carrying a glass tip and provided with a screw clip. The material is kept in position in the tube with a loosely packed plug of glass-wool. Before being treated with the tissue extract, the adsorbent is washed with a total volume of approximately 100 ml. of distilled water. The final washings must give no blank when allowed to react with 5 ml. 0.01 *M* HIO_4 for 10 min. at room temperature. To prevent the formation of air bubbles in the column, which would impede the passage of liquid, the tube should contain distilled water into which the adsorbent is poured, the level of the liquid being kept above the top of the adsorbent. After the adsorbent has been washed sufficiently, the supernatant liquid from the yeast treatment is poured into the tube and allowed to drip through the column at a rate such that the whole of the liquid passes through in approximately 90 min. When the liquid has passed through completely, the first 10 ml. portion of the yeast washings is poured into the tube, and the rate of flow adjusted so that this liquid passes through in approximately 15 min. The tube containing the column of adsorbent is sucked dry on a filter pump, and then a second 10 ml. portion of yeast washings is allowed to drip through the column, which is then sucked dry. The column is washed with the help of suction with 15 successive 5 ml. portions of distilled water. This amount of washing is found to be necessary and sufficient to obtain quantitative recovery of 1 mg. of added inositol.

The assumption has been made that the only HIO_4-reacting substances which are not removed from yeast-fermented tissue (muscle) extracts by the adsorbent mixture are inositol and glycerol.

Differential oxidation of inositol and of glycerol by HIO_4. The percentage oxidation of glycerol (0.8 mg./100 ml.) by 2 ml. 0.01 *M* HIO_4 at 8° C. at intervals up to 100 min. is as follows:

Time in min.	20	30	40	60	70	80	90	100
Per Cent Oxidation	63.0	68.6	79.8	95.6	98.0	100	100	100

The time for complete oxidation of glycerol depends on the temperature at which the reaction is carried out and on the concentration of both glycerol and HIO_4; for example, at 6° C. 1 mg. of glycerol in 10 ml. water is completely oxidized by 3.0 ml. 0.01 *M* HIO_4 in 15 min.

If inositol is present, approximately only 2% of the total inositol content (in the range of 0.5-2.5 mg.-%) will react with 2 ml. 0.01 *M* HIO_4 in 90 min. The reaction with inositol becomes, under the same conditions of concentration and temperature, complete in 48 hr. Thus, the reaction rate of inositol is much slower than that of glycerol. The difference of titration values obtained after 90 min. and 48 hr. multiplied by 1.02 gives the inositol content of the solution.

Estimation of Free Inositol. The effluent from the adsorption procedure is made up to 200 ml. in a graduated flask and 100 ml. withdrawn. Both portions are cooled to 8° C. To one portion are added 10 ml. 0.01 *M* HIO_4 (also at 8° C.) and the reaction is allowed to proceed at 8° C. for 90 min. If a temperature other than 8° C. is selected, the time relationships for oxidation of both glycerol and inositol at this temperature must be determined. The reaction is stopped in both cases by adjusting the pH to approximately 6.5 by the addition of 5 ml. phosphate buffer, followed by 5 ml. 5% KI solution. After 5 min., the I_2 liberated is titrated with approximately 0.004 *N* $Na_2S_2O_3$, with 1% soluble starch in saturated NaCl as indicator, the end-point being stabilized by the addition of a few drops of saturated $NaHCO_3$ solution. One mg. inositol contained in 100 ml. distilled water is also allowed to react with 10 ml. 0.01 *M* HIO_4 for 48 hr. at 8° C., and the excess HIO_4 titrated as above.

The HIO_4 reduced is obtained from the difference between the titration of the purified tissue extract after reaction with HIO_4 and the blank titration obtained with the HIO_4 alone.

Let X = HIO_4 reduced in terms of 0.004 *N* $Na_2S_2O_3$ in 48 hr. at 8° C.
Y = HIO_4 reduced in terms of 0.004 *N* $Na_2S_2O_3$ in 90 min. at 8° C.
Z = HIO_4 reduced by 1 mg. inositol in 48 hr. at 8° C.

The approximate inositol content in mg. of half the extract is given by $(X\text{-}Y)/Z$ which should be multiplied by 1.02.

Estimation of "Combined Inositol." For the estimation of the water-soluble combined inositol, the extract is hydrolyzed with acid (275). Both water soluble fractions (*i.e.*, "free" and "combined") are determined in the sample of tissue, with twice as much tissue as in the procedure outlined above. Before fermentation, the extract is made to 50 ml., and one-half is refluxed for 6 hr. with HCl (final concn. 18% HCl). The HCl is removed by distillation under reduced pressure, and the contents of the flask are washed out with distilled water to give a total volume of approximately 25 ml., and then neutralized by the dropwise addition of *N* NaOH. The difference of inositol values found in the two samples gives the amount of "bound" inositol in the test material.

X. *p*-Aminobenzoic Acid

p-Aminobenzoic acid, like sulfanilamide and related sulfa compounds, is a primary arylamine. The method of Marshall (276-278), used for the determination of sulfanilamide, is equally applicable to the quantitative estimation of *p*-aminobenzoic acid (279). It consists in coupling a diazotized solution of *p*-aminobenzoic acid with dimethyl-α-naphthylamine (279) or with N-(1-naphthyl)-ethylenediamine dihydrochloride (280). The resulting red color is measured in a photocolorimeter.

Apparatus.

A Klett-Summerson photoelectric colorimeter with green filter No. 56 and matched test tubes of 12.5 mm. diam. (or any other standard photoelectric colorimeter with appropriate filter).

15 × 150 mm. lipped Pyrex test tubes graduated at 10 ml. and 12 ml.

Reagents.

Sodium nitrite, 0.2%, freshly prepared.

Hydrochloric acid, approximately 4 *N*.

Ammonium sulfamate (La Motte Chemical Products Co., Baltimore, Md.), 2%. This is a much stronger solution than is necessary, but assures complete destruction of excess nitrite during the analysis.

N-(1-naphthyl)-Ethylenediamine Dihydrochloride (La Motte). 100 mg. are dissolved in 100 ml. of water and preserved in a dark bottle.

Trichloroacetic acid (Merck), 15 and 2.7% solutions. The latter is conveniently prepared by diluting 18 ml. of the 15% solution to 100 ml.

p-Aminobenzoic acid (Eastman), recrystallized, 100 mg. dissolved in 100 ml. of 2.7% trichloroacetic acid. From this stock solution dilutions are made for standards.

Quantitative determination of *p*-aminobenzoic acid is used mainly in blood, urine, in broth cultures, or other bacteriological media, in extracts prepared from yeast, liver, rice bran, and in similar products. Detailed description of the procedure to be used will be given for blood and broth cultures. With proper modification the same technique may be applied to other test materials.

Preparation of Blood Filtrates. Thirty ml. of distilled water and 2 ml. of oxalated or citrated blood are thoroughly mixed in a 50 ml. flask. For completion of hemolysis the mixture is allowed to stand for about 5 min. Eight ml. of 15% trichloroacetic acid are added slowly with rotation, whereafter the mixture is shaken vigorously, again allowed to stand for 15 min. and filtered through filter paper.

Ten ml. of the filtrate are placed in a 50 ml. flask, followed by two ml. of water and 1 ml. of 0.1% solution of $NaNO_2$. The mixture is allowed to stand for from 15 to 20 min. Then 1 ml. of 2% ammonium sulfamate is added and again allowed to stand for from 2 to 3 min. Finally, 5 ml. of N-(1-naphthyl)-ethylenediamine dihydrochloride solution are added and the color developed is read after 30-60 min. in the colorimeter with the blank set at zero. The blank for this determination consists of 10 ml. of 2.7% trichloroacetic acid treated exactly as above.

p-Aminobenzoic acid may be present in body fluids, such as blood and urine, not only in free but also in conjugated (acetylated) form. Conjugated *p*-aminobenzoic acid will not give the color reaction described above, and requires preliminary treatment with acid for its determination. This is carried out by adding 0.5 ml. of 4 *N* HCl to 10 ml. of the blood filtrate in a graduated Pyrex test tube. The tube is placed in a boiling water bath for 1 hr., cooled to room temperature, and the mixture diluted with water to the 10 ml. mark. The solution is now treated exactly as in the case of the free *p*-aminobenzoic acid.

Determination of p-Aminobenzoic Acid in Media. For the determination of *p*-aminobenzoic acid in broth cultures, the cultures are passed through a porcelain filter and 2 ml. samples of the filtrate mixed with 8 ml. of distilled water and 2 ml. of 15% tricholoracetic acid. The determination is carried out as described under blood filtrates. The sample is centrifuged until clear and is then ready to be read in the colorimeter with a suitable blank set at zero.

Color production by *p*-aminobenzoic acid, with its maximum at 545 mμ, follows Beer's law over the concentration range 0.0025-0.05 mg./100 ml.

Calculation may be based on a standard reference curve drawn from data as obtained by analyzing solutions of pure *p*-aminobenzoic acid of known strength.

With the Klett-Summerson photoelectric colorimeter, readings are directly proportional to the concentration of the substance being determined; the factor equals the amount of standard divided by the scale reading. As standard, a solution containing 0.025 mg. of *p*-aminobenzoic acid may be used. It is treated exactly as described above. To calculate free *p*-aminobenzoic acid, the scale reading is multiplied by the factor. Conjugated *p*-aminobenzoic acid is determined by comparing the amounts found before and after hydrolysis.

The specificity of this diazotization method is limited. A large number of chemicals, such as phenol, cresol and all primary arylamines, some of the latter of biological significance, will give color reactions by the method.

A greater degree of specificity may be obtained by chromatographic separation of *p*-aminobenzoic acid in its diazotized form (281). The precision of the method in its quantitative aspect has not yet been established. As a more qualitative procedure, it is carried out as follows (281): The diazotized compounds are coupled with an alcoholic solution (1 ml. in 250 ml. of 95% ethanol) of dimethyl-α-naphthylamine (Eastman) and the azo dyes extracted with ether. The dyes derived from aromatic amino acids are extracted from ether by dilute Na_2CO_3, while non-acidic dyes and excess amine remain in ether. The acidic dyes are brought back into fresh ether by gradual acidification with H_3PO_4 and "chromatographed" on alumina (Merck-Brockmann for chromatographic analysis). The dyes are adsorbed in the top layer. Development with ether containing 2% acetic acid washes the *p*-aminobenzoic acid dye down the column as a sharp orange-yellow ring. Presence of anthranilic acid is at once indicated by the formation of a deep purple ring which moves down the column much more slowly and is eluted by ether containing 20% acetic acid. *m*-Aminobenzoic acid is not readily separated from *p*-aminobenzoic acid by this method. The amount of the dyes is estimated by extracting from ether with 20% aqueous HCl, and photocolorimetric or spectrophotometric estimation of the red-violet solution at a wavelength of 530 mμ.

XI. Pteroylglutamic Acid and Related Compounds of the Folic Acid Group

Pteroylglutamic acid and related compounds of the folic acid group containing *p*-aminobenzoic acid in peptide-like linkage, on either chemical or catalytic reduction at an acid pH, are cleaved to yield *p*-aminobenzoylglutamic acid. The reaction seems to be general for this group of compounds, and is suggested (282) as a basis for a chemical method for the determination of pteroylglutamic acid, its derivatives, and analogues.

The method consists of reducing the compound with zinc dust in 0.5 *N* HCl. The aromatic amine is measured by the method of Bratton and Marshall (278) before and after reduction. The difference between the 2 values multiplied by the appropriate factor is a measure of the pteroyl derivative present.

As the sensitivity of the colorimetric procedure is not great, this chemical method is of no value in determining the pteroylglutamic acid content of natural materials unless the active compound is present at a concentration sufficient to give a readable color.

Reduction of pteroylglutamic acid and similar compounds is carried out with the help of zinc dust. The following example should illustrate the procedure: A solution containing from 0.5 to 3.0 mg. of pteroylglutamic acid (or an equivalent amount of similar compounds) is placed in a 100 ml. volumetric flask. To this flask are added approximately 80 ml. of water, 10 ml. of 5.0 *N* HCl, 1.0 ml. of 0.5% solution of gelatin, and sufficient water to make the total volume 100 ml. A sample of this unreduced solution, usually 2 ml., is removed and used for determining the free amine. If the original compound or crude product contains less than 2% of free amine, an aliquot of the original solution must be used in order to obtain a readable color.

The remainder of the solution is transferred to a 250 ml. Erlenmeyer flask and reduced by the addition of from 0.5 to 1.0 g. of zinc dust. The amount of zinc dust is not critical and can be conveniently estimated once a weighed amount has been used. After 10 min. reduction, during which time the flask is shaken intermittently, the zinc dust is filtered off and the amine is determined on 2 ml. of the filtrate following the Bratton and Marshall method ((278), see p. 256). *p*-Aminobenzoic acid is used as standard and a response curve is constructed with from 5 to 20 γ of *p*-aminobenzoic acid. This is necessary since the color intensity does not exactly follow Beer's law.

The difference obtained between the amine obtained after reduction and the amine obtained on the unreduced solution yields the combined amine present. This value multiplied by the factor, (molecular weight of pteroylglutamic acid)/(molecular weight of *p*-aminobenzoic acid) gives the micrograms of pteroylglutamic acid in the diluted sample. When the method is used for the various derivatives or analogues, the desired factor is obtained from the following ratio, (molecular weight of analogue)/(molecular weight of *p*-aminobenzoic acid).

The method is satisfactory with concentrates derived from natural sources when the content of the active compound is 5% or greater. The method is entirely satisfactory for determining the potency of crude products derived from various synthetic reactions when only the naturally occurring isomer is present.

The biological (Vol. II) and especially the microbiological (pp. 395, 490) methods are far superior to this chemical method with regard to their respective sensitivity, and should be given preference in assay work.

XII. Ascorbic Acid

1. Historical Development

Ascorbic acid is easily and reversibly oxidized to dehydroascorbic acid. The two forms of the vitamin exhibit equal antiscorbutic activity. While the predominant form of the vitamin in most materials is reduced ascorbic acid, dehydroascorbic acid is present in a number of natural materials in sufficient amounts, and is formed under such various circumstances during processing and storage, that the determination of this form is often desirable. Nearly all the methods that have been proposed for the determination of ascorbic acid involve reversible oxidation to dehydroascorbic acid. The methods for the determination of the latter are based either on direct formation of a color complex or on preliminary reduction to ascorbic acid.

A. REDUCED ASCORBIC ACID

Many methods for the colorimetric determination of ascorbic acid have been proposed (1, pp. 316-322; 283, pp. 366-379), but only 3 color reactions have achieved widespread usage. Titration with a standardized solution of iodine (*cf.* 1, p. 316) has been found applicable only to fairly pure solutions of ascorbic acid and to some high potency pharmaceuticals. Reaction with methylene blue (284-286) has been used by some investigators for clinical work. The reaction which has been chiefly exploited for quantitative assay is that between ascorbic acid and the dye 2,6-dichlorobenzenoneindophenol (dichlorophenolindophenol). To measure the extent of these reactions, and thereby the concentration of ascorbic acid in the extract under investigation, visual, electrometric, and photometric procedures have been devised. All 3 types of measurement are illustrated in the section on methods.

Following the observation of Zilva (287) that the antiscorbutic fraction of lemon juice reduced phenolindophenol to its leuco form, Tillmans and his group (288-295) proposed and developed a method based on visual titration of ascorbic acid with a standardized solution of 2,6-dichlorophenolindophenol. Numerous modifications of this visual procedure resulted from the investigations of other workers (*cf.* 283, pp. 367-370); among other conclusions, it was established (296-298) that metaphosphoric acid was most suitable for the extraction of the vitamin from plant materials. It soon became apparent, however, that, besides ascorbic acid, many substances present in plant tissues and other materials reduce 2,6-dichlorophenolindophenol to its leuco form, although most interfering substances react more slowly with the dye (*cf.* 299).

The recognition of this fact, coupled with the availability of photoelectric colorimeters, led to the evolution of photometric methods which attempted to overcome interference by other reducing substances. Bessey (300) proposed a method which utilized measurements of the intensity of the dye color 15 and 30 sec. after addition of the unknown extract to the dye, and subsequent extrapolation of these values to zero time by a simple formula to obtain a measure of the reaction of ascorbic acid alone with the dye; moreover, his procedure eliminated the necessity for absolutely clear and colorless filtrates. Evelyn, Malloy and Rosen (301) similarly increased the specificity and accuracy of the method by charting the course of the reaction after addition of an excess of dye to the extract; graphic extrapolation to zero time of points obtained at 5, 10, 20 and 30 sec. was used to obtain the true (*i.e.*, immediate-reacting) ascorbic acid. Hochberg, Melnick and Oser (302) later made use of a similar extrapolation of 5 and 10 sec. readings in their photometric method. Modifications based on the reaction of H_2O_2 with SO_2 prior to colorimetry (303), and on titration in acetic acid (304) rather than metaphosphoric acid, have been proposed to eliminate interference from 2 common reducing substances, SO_2 and ferrous iron.

A different approach to the problem of ascorbic acid determination was provided by the use of electrometric rather than visual or photoelectric measurement of the end point. This procedure, which is based upon the rapid change of e.m.f. which occurs at the end-point of the titration, is useful when highly colored or turbid extracts are to be analyzed; it does not, however, offer any correction for the presence of any interfering substances which react with the oxidant (iodine or 2,6-dichlorophenolindophenol) rapidly enough to exert a noticeable effect during the time needed to reach the end-point. In this respect, electrometric titration is preferable to visual titration but does not represent any improvement over the photometric method.

Several methods of analysis have been developed on the electrometric principle. Following the mention of this possibility by Birch and coworkers (305), an electrometric titration method was proposed by Becker and di Gleria (306). These authors made use of a platinum plate as measuring electrode, while the saturated calomel electrode served as the standard electrode. The titration was carried out in a saturated $Al_2(SO_4)_3$ solution and the readings plotted. Kirk and Tressler (307) employed an electrometric titrimeter with a bimetallic electrode of platinum and tungsten in a titration medium of 8% acetic acid. They (307) claimed that plotting the titration curve was more accurate than determination of the end-point by means of the blink of the electric eye. Harris and coworkers (308) pointed out that in both these methods the titration curves show a continuous drift in potential throughout the whole course of the titration with the dye, which makes it impossible to locate the end-point with accuracy. These investigators then developed an electrometric titration method which employed a mercury-platinum electrode, consisting of a mercury-coated platinum wire. An alternative procedure to be used in the presence of high concentrations of interfering reducing substances involved the use of a polarized bright platinum electrode. This method is presented in detail in the section on methods. Adams, Acker and Frediani (309) recently described a titrimetric determination of ascorbic acid in which iodine was employed as the reagent and the measurements were made with a vacuum tube potentiometer (titrimeter) utilizing a polarized platinum-platinum electrode system.

A different approach to the problem of specificity of the colorimetric reaction was provided by the work of Folkman (310), who proposed a method of assay based on the fact that oxidized 2,6-dichlorophenolindophenol could be quantitatively extracted from an acid solution by xylene. Bukatsch (311) confirmed and extended this work. Stotz (312) made use of these findings in developing a clinical method of considerably increased accuracy and speed. Highet and West (313) proposed the use of a standardized xylene solution of the dye rather than extraction of the unreduced dye from the aqueous layer. However, Pepkowitz (314) concluded, from a study of both procedures, that the method of Stotz was to be preferred for a number of reasons, and adapted the latter method to plant materials. Recently, Robinson and Stotz (315) presented an extension of this procedure, which improves the specificity of the method for a variety of natural materials. Although the rapid extraction with xylene removes the unreduced dye from contact with many reducing substances, several types of compounds react with the dye fast enough to introduce considerable error at times. Reductones, formed by the degradation of carbohydrates in alkali (316), and reductic acid, formed by heating carbohydrates in acid (317), behave toward oxidizing agents, including 2,6-dichlorophenolindophenol, very much like ascorbic acid. These interfering substances have been found in such foods as evaporated milk, dehydrated cabbage, unenriched fruit juices, jellies, and other fruit products. In general, these are often encountered in materials which have been subjected to high temperatures or long storage. Robinson and Stotz adopted the formaldehyde technique developed by Lugg (318), and Snow and Zilva (319), to minimize the interference caused by these substances.

To eliminate interference from sulfite, which may occur in sulfite-treated foods, and of reduced iron or tin, which are often found in canned products, especially after long storage, Robinson and Stotz included the H_2O_2 treatment of Levy (303). The details of these modifications are described in the methods section.

B. DEHYDROASCORBIC ACID

To determine dehydroascorbic acid, most investigators convert this form to reduced ascorbic acid prior to determination of the latter. This conversion is accomplished by bubbling H_2S through the extract containing dehydroascorbic acid; the H_2S is removed by bubbling wet nitrogen gas through the solution after the H_2S treatment. This procedure is not above criticism. King (320, 321) pointed out that treatment of certain substrates with H_2S resulted in the formation of reducing substances which interfered in the colorimetry. Hochberg, Melnick and Oser (302) tried to minimize these interferences by their more rapid photometric measurement.

To overcome the difficulties inherent in the H_2S treatment, Roe and coworkers (322, 323) developed a method based on a direct colorimetric determination of dehydroascorbic acid. The oxidized form of the vitamin, in a metaphosphoric acid extract, is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative is treated with H_2SO_4 to produce a red color which is measured photometrically. If the determination of total ascorbic acid (reduced and oxidized forms together) is desired, reduced ascorbic acid is first oxidized to dehydroascorbic acid by treatment with Norit. If only the dehydro form is to be determined in the presence of reduced ascorbic acid, thiourea is added to prevent the oxidation of the latter.

Bolomey and Kemmerer (324) modified the method of Roe and Kuether (322) by substituting glacial acetic for 85% H_2SO_4 in the color development step. The advantages claimed are that charring is eliminated, less care required, and much time saved. In a second paper (325), Bolomey and Kemmerer, published spectrophotometric studies with sulfuric and acetic acids in the absence of thiourea showing that Norit oxidation of citrus juice solutions causes less distortion of the spectrophotometric absorption curves than does oxidation with bromine. In a critical study of the modifications proposed by Bolomey and Kemmerer (324, 325), Mills and Roe (326) have presented spectrophotometric data which show that these modifications may impair the specificity of the original procedure, since the apparent superiority of H_2SO_4 over acetic acid rests upon the fact that H_2SO_4 is a reactant as well as a solvent. The importance of thiourea is stressed as a means of preventing the effect of oxidants and controlling the rate of coupling in the color development reaction.

The Roe method (322-323) is included among those presented in detail in the methods section. It should be noted, however, that the method is not yet free from objections. Reactions with 2,4-dinitrophenylhydrazine have been reported for phenylpyruvic acid (327) and other α-keto acids (328-330). Penney and Zilva (331) described the reaction of 2,3-diketo-L-gulonic acid, a compound likely to be formed from dehydroascorbic acid by mutarotation and loss of lactone structure, with this color reagent and showed (332) that this compound, as well as D-glucoascorbic acid and D-araboascorbic acid, cannot be distinguished from L-ascorbic acid by this method. Pijoan and Gerjovich (333) found that, in natural materials, such as orange juice which had undergone unpredictable antecedent oxidation, the "total ascorbic acid" was erroneously very high compared to the actual vitamin C content as determined biologically; this finding was confirmed by similar data obtained with synthetic acid. On the basis of these data and of the previously mentioned earlier investigations, these authors (333) concluded that the 2,4-dinitrophenylhydrazine method may give unreliable results for the antiscorbutic value of certain biological preparations if loss in lactone structure of dehydroascorbic acid has taken place.

These observations indicate the need for further elaboration and improvement of this method. At the present time, the H_2S procedure is still most generally employed for the estimation of dehydroascorbic acid in a majority of materials (334).

Stewart and Sharp (335) have presented a unique method based on the enzyme-catalyzed oxidation of ascorbic acid and interfering substances by concentrated cucumber juice (ascorbic acid oxidase) with subsequent specific reduction of dehydroascorbic acid to ascorbic acid by a suspension of Escherichia coli or Staphylococcus albus. The ascorbic acid formed in this fashion is then determined by titration with the usual indophenol dye.

2. Methods of Analysis

The following procedures are described in detail.

1. Visual Titration with Iodine, based on the description for iodometry of Kolthoff and Sandell (336, pp. 584-596). This procedure is applicable, in general, to high-potency preparations (*e.g.*, pharmaceuticals) which do not contain appreciable amounts of other reducing substances.

2. Visual Titration with 2,6-Dichlorobenzenoneindophenol, based on the procedure of the U. S. Pharmacopeia (337). This procedure is trustworthy for fruits and vegetables which contain significant quantities of ascorbic acid. It can also be used in place of iodometric titration wherever the latter is applicable.

3. *Electrometric Titration with 2,6-Dichlorobenzenoneindophenol,* as described by Harris and coworkers (308) especially for turbid or highly colored extracts.

4. *Photometric Indophenol-xylene,* based on the method of Robinson and Stotz (315), with corrections for various interferences. This procedure is capable of widespread application and is recommended for routine practice wherein highly diversified types of samples are encountered.

5. *Reduction of Dehydro- to Ascorbic Acid* with H_2S (334).

6. *Direct Colorimetric Determination of Dehydroascorbic Acid* with 2,4-dinitrophenylhydrazine, Roe *et al.* (322, 323).

A. VISUAL TITRATION WITH IODINE

Reagents.

Iodine, 0.1 *N*. Weigh 12.7 g. of reagent quality iodine into a 250 ml. beaker, add 40 g. of iodate-free KI and 25 ml. of water. Stir at intervals to hasten solution of the iodine, and when all has dissolved dilute to 1000 ml. Filter into an amber glass-stoppered bottle and store in a cool place. See below for standardization. Protect the solution from light as much as possible, and keep the stock bottle well stoppered.

Starch Solution. Prepared every 2-3 weeks by boiling about 7 g. of corn starch in approximately 1 l. of water and filtering through cheesecloth.

Arsenious oxide, 0.1 *N*. Weigh 2.2474 g. of pure dry arsenious oxide (obtained from the Bureau of Standards, or supplied by various manufacturers, and dried in a desiccator over H_2SO_4) into 20 ml. of 1 *N* NaOH. H_2SO_4 or HCl (1 *N*) is added to the solution until the reaction is neutral or only slightly acid to litmus paper; the solution is transferred to a 500 ml. volumetric flask and made to volume with water.

Hydrochloric acid, 5 *N*. Prepared by dilution from concentrated HCl (12-13 *N*).

Procedure.

Standardization of 0.1 N Iodine. Exactly 25 ml. of 0.1 *N* standardized arsenious oxide is pipetted into a 250 ml. Erlenmeyer flask, 25-50 ml. of water, 1.0 g. of $NaHCO_3$ and a few ml. of starch solution are added, and iodine is gradually added from a burette until the first blue color is obtained. The exact normality of the iodine solution is obtained from

$$\frac{0.1 \times 25}{\text{ml. iodine}} = \text{Normality of iodine.}$$

Assay.

A sample containing 100-200 mg. of ascorbic acid is usually used;

solids are dissolved in water, liquids are diluted as required. The total volume of the sample (with or without added water) should not exceed 150 ml. The sample is placed in a 250 ml. Erlenmeyer flask; a few drops of 5 *N* HCl and 10-20 drops of starch solution are added. Standardized 0.1 *N* iodine is gradually added from a burette until the first stable blue color appears.

To obtain the factor needed to convert ml. of iodine to ml. of ascorbic acid, the following procedure is followed. Exactly 0.1 g. of U.S.P. Ascorbic Acid Reference Standard is weighed into a 250 ml. Erlenmeyer flask and covered with about 100 ml. of water. A few drops of 5 *N* HCl and 10-20 drops of starch solution are added and the solution is titrated with the standardized 0.1 *N* iodine solution. If it is found, for example, that 11.35 ml. of 0.1 *N* iodine solution are needed to titrate a solution containing 100.0 mg. of ascorbic acid, then 1.00 ml. of 0.1 *N* iodine is equivalent to 8.8 mg. of ascorbic acid.

Then, ml. iodine needed to titrate unknown × 8.8 = mg. ascorbic acid in unknown.

B. VISUAL TITRATION WITH 2,6-DICHLOROBENZENONEINDOPHENOL

Reagents.

Metaphosphoric Acid Solution. Stock solution (20%). Dissolve with shaking 100 g. of reagent grade metaphosphoric acid in about 400 ml. of distilled water and dilute to 500 ml. Filter rapidly through a fluted filter paper into a glass-stoppered bottle. This stock solution may be used for about a month if kept refrigerated.

Working solution (4%). Dilute 1 part of stock solution with 4 parts of distilled water. This working solution may be used for several days if it is kept refrigerated.

Sodium 2,6-Dichlorobenzenoneindophenol Solution. Dissolve 100 mg. of the powdered dye (Eastman Kodak) in 100 ml. of warm distilled water, cool, dilute to 500 ml. and filter through a fluted paper into an amber, glass-stoppered bottle. The solution may be used for 10-14 days if stored in the refrigerator.

Procedure.

Standardization of the Dye. Accurately weigh 0.1 g. of U.S.P. Ascorbic Acid Reference Standard, transfer it to a 100 ml. volumetric flask and make up to volume with 4% metaphosphoric acid. Immediately transfer 2 ml. of the ascorbic acid solution to a 50 ml. Erlenmeyer flask containing 5 ml. of 4% metaphosphoric acid, and titrate rapidly with the dichlorophenolindophenol (hereinafter designated as DI solution) until a distinct rose-pink color persists for at least 5 sec. The concentration of the DI solution is expressed as mg. of ascorbic acid equivalent to 1 ml. of DI solution.

Assay Procedure. A representative weighed sample is placed in a Waring blendor with 400-500 ml. of 4% metaphosphoric acid and blended for one to a maximum of 3 min. The entire contents are transferred quantitatively to a 1 l. volumetric flask and diluted to mark with distilled water. The extract is mixed well and filtered through a coarse fluted paper. If necessary, subdilutions are made in 4% metaphosphoric acid. Liquids such as fruit juices can be diluted with 4% metaphosphoric acid without blending. The final extract in all cases should contain between 0.1 and 0.4 mg./ml. Ten ml. aliquots of the final extract are pipetted into 125 ml. Erlenmeyer flasks and titrated rapidly with the standardized DI solution. The titration values are averaged and the ascorbic acid content calculated according to

$$S \times E \times \text{dilution factor} = \text{mg. ascorbic acid per sample,}$$

where S = ml. DI solution used to reach the end point,
E = mg. ascorbic acid equivalent to 1 ml. DI solution.

Notes on the Procedure.

1. Interference by reduced iron, tin, sulfite, *etc.*, can be eliminated by adding to the aliquot of metaphosphoric acid an equal volume of 3% H_2O_2 just before titration with the dye. The remainder of the procedure is the same.

2. Schwarze and Guenther (338) determined blank titrations after destroying ascorbic acid by adding copper salts to the metaphosphoric-hydrochloric acid extracts.

C. ELECTROMETRIC TITRATION WITH 2,6-DICHLOROBENZENONEINDOPHENOL

Reagents.

Metaphosphoric acid solution.

Stock solution (20%). As described under B.

Working solution (5%). Dilute 1 part of stock solution with 3 parts of distilled water. This working solution may be used for several days if it is kept refrigerated.

Sodium 2,6-Dichlorobenzenoneindophenol Solution. Dissolve 40 mg. of the powdered dye (Eastman Kodak) in 40 ml. of hot boiled distilled water and filter. Cool to room temperature, add KCl to make a final concentration of 0.046 *N*, and make up to 50 ml. Standardize by direct titration, (see details under B), against a solution of pure ascorbic acid of known strength. One ml. of the dye solution should be equivalent to 0.4 mg. of ascorbic acid.

Potassium Chloride. Saturated solution.

Acid-washed Sand.

Apparatus.

Potentiometric Assembly. Consists of a saturated KCl-calomel half-cell, with the calomel end connected to the negative pole of a potentiometer and the KCl sidearm dipping into a beaker with saturated KCl; an agar bridge connecting this beaker to a reaction tube in which the titration takes place; and a needle electrode (see description below) which dips into the reaction tube and is connected to the positive pole of the potentiometer.

Mercury-Platinum Double Electrode. Prepared as follows. An ordinary bright platinum electrode with the platinum wire protruding about 1 cm. below the seal made of cobalt glass is cleaned with warm 6 *N* HNO_3, followed by alcoholic KOH, and rinsed with distilled water. It is plated by being immersed in about 100 ml. of a 1% solution of $HgCl_2$ for 30-60 sec. with the application of a potential of 1.5 V. from a dry battery, the electrode being made the cathode while another platinum electrode 1-2 cm. distant serves as the anode. After plating, it is washed with distilled water and is then ready for use. The electrode may generally be used a number of times during a day's work without replating, but should be replated at the start of each day's work.

Microburette. Graduated in 0.01 ml.

Inlet tube connected to a tank of N_2 or CO_2 which is bubbled into the reaction tube to agitate the extract during titration.

Procedure.

Weigh out 5-10 g. of the material to be analyzed (10 g. for a material of low, and 5 g. for one of high potency), and cover it with 5% metaphosphoric acid in a mortar. Add about 2-4 g. of acid-washed sand and grind the material to a fine pulp. Centrifuge and wash the residue twice with 10-15 ml. of the acid. Combine the clear centrifugates and make up to 50 ml. or more depending on the concentration of the vitamin. A suitable dilution is about 4 mg. of ascorbic acid/100 ml., so that 10 ml. of extract will require 1-2 ml. of the standard dye solution. The use of less than 0.5 ml. or more than 4 ml. of the latter is not recommended.

Measure by pipette 10 ml. of the extract into the reaction tube and add 0.1 ml. of a saturated solution of KCl, so that the final concentration of the latter is about 0.046 *N*. With the electrode and agar bridge in position, pass a stream of N_2 or CO_2 into the solution at a rapid, constant rate. After about 30 sec., take the reading of e.m.f. Add from the burette 0.01 ml. or more of the dye and take the reading again after 15 sec. Proceed in this way until the end-point is reached, as indicated by a sharp rise of e.m.f. At this part of the curve, wait for 45-60 sec. before taking each reading. Carry on the titration a little beyond the end-point until the e.m.f. shows little further change with each addition of the dye, *i.e.*, the curve begins to flatten out once again. It is advisable to run a preliminary rapid titration first to ascertain the approximate amount of the dye needed and then do a more careful titration. In this second titration, run in the dye rapidly to within 0.1 ml. of the volume required according to the preliminary finding, and then add slowly 0.02-0.03 ml. at a time, until the exact end-point is reached, again waiting 45-60 sec. before taking each reading on the rising part of the curve.

Plot the results with the e.m.f. as ordinate and the volume of dye added as abscissa, and draw a smooth curve through all the points. Draw two tangents, one along the base line (which should be around 100-120 mv. for solutions of pure ascorbic acid and a little lower for some natural extracts), and the other along the steepest part of the rising e.m.f. The end-point is taken as the point of intersection of the two tangents.

Alternative Electrode for Use with High Concentrations of Interfering Reducing Substances. When the concentration of interfering reducing substances is unusually high in comparison with the amount of ascorbic acid present, as in liver and kidney, the slight sluggishness at the end-point constitutes a serious disadvantage owing to the slow oxidation of the interfering substances during the minute of waiting.

When working with such materials, it is preferable to rely on a modified use of the bright platinum electrode, which, in spite of its many disadvantages, does give a very quick response at the end-point. To avoid drifting of potential in the preliminary phase of titration, the contact key is tapped 2 or 3 times after each addition of the dye so as to polarize the electrode. In this way, the initial e.m.f. can be kept more or less constant until the end-point is reached, when, in the presence of excess redox dye, the electrode is no longer polarizable and establishes an equilibrium immediately with the solution. The initial potential of the electrode is kept low by treating the electrode, before use, with alcoholic KOH or alkaline pyrrogallate after the cleaning with HNO_3. This step is necessary as otherwise the e.m.f. might be too high and the change at the end-point scarcely noticeable. It is understood, of course, that, in using the platinum electrode in this way, the initial potentials are not true equilibrium values. However, this does not influence the results appreciably, as long as the initial e.m.f. is kept low.

Notes on the Procedure.

1. The method has been applied by the authors to many vegetables and fruits, as well as to animal tissues. On these products, the method gave values which agreed to within 1-2% with a visual titration method.

2. In control tests, ascorbic acid could be recovered with an error of less than 0.5-2.0% at concentrations of 0.2-0.02 mg./ml., respectively.

3. If the measurement of dehydroascorbic acid is desired, this form is first reduced to ascorbic acid as described below under E.

D. PHOTOMETRIC INDOPHENOL-XYLENE PROCEDURE

Reagents.

Metaphosphoric acid solutions. As described under B.

Sodium 2,6-Dichlorobenzenoneindophenol. As described under B.

Acetate Buffer. Dissolve 250 g. of crystalline sodium acetate (trihydrate) in 250 ml. of water, filter, add 500 ml. of glacial acetic acid and dilute to 1 l. The pH should be 3.9-4.0.

Xylene. Many lots may be used without treatment, but some lots must be redistilled (*cf.* 293). The waste xylene resulting from the determinations may be collected, washed with dilute alkali, purified by distillation, and used again.

Hydrogen peroxide (3%). Dilute 30% C.P. H_2O_2 10-fold. Keep refrigerated. Prepare weekly.

Formaldehyde. Technical grade, 40% solution.

Hydroquinone. Add an equal amount of acetone to a saturated solution of hydroquinone in acetone, to obtain a half saturated solution. This must be prepared fresh before use.

Apparatus.

A *Photoelectric Colorimeter* with 500 mμ filter (the 490 mμ filter of the Evelyn colorimeter is also satisfactory).

A Waring Blendor.

Centrifuge Tubes, 50 ml. heavy duty, glass-stoppered or fitted with cork or rubber stoppers.

Syringe, Yale Luer Lok type, 20 ml. capacity.

Procedure.

A weighed sample or suitable aliquot of a liquid containing at least 1 mg. of ascorbic acid is placed in a Waring blendor containing metaphosphoric acid solution. Liquid samples and solids may be blended in a mixture of 20% HPO_3 and water, the aliquot of 20% HPO_3 being $\frac{3}{20}$ of the volume to which the blending is to be diluted; thus, the final HPO_3 concentration will be 3%. The mixture is blended for the minimum length of time necessary to obtain a homogeneous extract, usually about 2 min. This blending is transferred to a suitable volumetric flask, usually of 500 or 1000 ml. capacity, and made up to volume with distilled water. The mixture is shaken well and filtered immediately through Whatman No. 12 fluted paper, the first few ml. of filtrate being discarded.

If a subdilution is necessary, it is made in 3% HPO_3 at this point. The final extract may contain from 2 to 100 γ/ml.

A suitable aliquot (preferably 2-5 ml.) of the final extract, containing 20-100 γ of ascorbic acid, is pipetted into a 50 ml. centrifuge tube (preferably glass-stoppered). The acetate buffer is added at a ratio of at least 1 ml. buffer to 5 ml. of 3% HPO_3 extract. *Without delay,* 5 ml. of DI solution and 15 ml. of xylene are added. It is expedient to add the xylene from a previously loaded Luer Lok syringe. The tube is immediately stoppered and shaken vigorously for 10 sec. to extract the unreduced dye. If all the dye is decolorized, a smaller aliquot of the final extract is taken or a subdilution of the extract made before colorimetry. The tubes are centrifuged for several minutes at about 2500 r.p.m. to separate the supernatant xylene layer. The dye is then stable for several hours if kept out of direct light. Approximately 10 ml. aliquots of the supernatant xylene layers are pipetted into colorimeter tubes.

A duplicate set of "total dye" tubes is prepared for each set of unknowns by replacing the aliquot of final extract by acetate buffer and water carrying these tubes through the same operations as the simple tubes.

A *calibration curve* for the instrument is obtained in the following way. Exactly 0.1 g. of pure U.S.P. Reference Standard Ascorbic Acid is weighed into a 100 ml. volumetric flask and made up to mark with 1% metaphosphoric acid. Exactly 5 ml. of this solution are diluted to 100 ml. with 1% metaphosphoric acid. Into a duplicate set of tubes, exactly 1, 2, 3 and 4 ml. are pipetted serially, providing a series of standards containing 0.05, 0.10, 0.15, and 0.20 mg., respectively. A volume of buffer equal to the aliquot of standard solution is added, followed by 5 ml. of DI solution, 15 ml. xylene, and the steps described above are carried out.

If color from the sample interferes with the reading, a blank is run exactly as the sample, except that 5 ml. of water are added in place of the 5 ml. of dye solution.

The following table summarizes the amounts of the various solutions added to the tubes.

Colorimetry. The colorimeter is set at 100% transmission with 10 ml. of pure xylene with the filter at 500 mμ, and the center setting is noted. The xylene extracts obtained from standard, "total dye" sample, and blank tubes are read against this center setting.

Removal of Interferences. If preliminary tests show that xylene removes color (other than the unreduced dye) from the acetate-buffered extract, then an additional operation is required with the sample tube. After the first reading, 2 drops of hydroquinone (in acetone) are added

Step No.	"Total dye" tubes	Standard tubes	Sample tubes	Blank tubes
1	5 ml. water	Graded volumes (1-4 ml.) of ascorbic acid solution	Aliquot of extract	Aliquot of extract
2	Volume of buffer equal to 1	Volume of buffer equal to 1	Volume of buffer equal to 1	Volume of buffer equal to 1
3	5 ml. of DI solution	5 ml. of DI solution	5 ml. of DI solution	5 ml. of water
4	15 ml. of xylene	15 ml. of xylene	15 ml. of xylene	15 ml. of xylene

to the xylene extract and mixed. The color due to excess DI is bleached within 30 sec., after which a second reading is taken to measure the blank due to the extract.

To eliminate the reducing action of sulfite, reduced iron or tin, peroxide is added prior to the dye. For this purpose, a volume of 3% H_2O_2 equal to the acetate-buffered extract, is added just before the dye. The remainder of the procedure is the same.

To correct for the action of reductones and reductic acid, the reducing action of ascorbic acid is selectively abolished by interaction with formaldehyde at pH 3.6-3.8 for 10 min. In practice, two tubes are required for the determination: tube 1 contains the selected aliquot of extract; tube 2, containing the same volume of extract, receives at least one-fifth that volume of acetate buffer, followed by enough 40% formaldehyde to give a final concentration of formaldehyde of exactly 8% (*e.g.,* 3 ml. of extract, 1 ml. of buffer, 1 ml. of 40% formaldehyde). The tubes are allowed to stand for 10 min. At the end of this period, tube 1 received its volume of acetate buffer and a volume of water equivalent to the volume of formaldehyde added to tube 2. 5 ml. of DI are then added to each tube and the excess dye extracted with 15 ml. of xylene as above. The difference between the amounts of dye reduced in the two tubes represents the true ascorbic acid (*cf.* note 1). Neither peroxide nor formaldehyde affects the stability of the dye in the xylene layer.

Calculations. The following calculations apply to Evelyn photoelectric colorimeter; similar calculations may easily be set up for other instruments.

All galvanometer readings are converted to photometric densities according to

$$L = 2 - \log G$$

The calibration constant, K, is obtained at the four levels of standard ascorbic acid run in the calibration from

$$K = \frac{L_t - L_s}{C_s}$$

where L_t = average photometric density of "total dye"
L_s = photometric density of standard
C_s = concentration of standard ascorbic acid per aliquot.

The average value of K is calculated from the values obtained at the four levels. The concentration of ascorbic acid in the unknown is obtained from

$$\frac{L_t - L_u + L_b}{K} \times \text{dilution factor} = \text{mg. of ascorbic acid/sample}$$

where L_t = average photometric density of "total dye"
L_u = photometric density of sample
L_b = photometric density for sample blank
K = average calibration constant.

Notes on the Procedure.

1. Robinson and Stotz (315) point out that, while this method is more specific than other procedures, there are some substances for which no adequate corrections are available or for which specialized methods are necessary, *e.g.*, no correction is available for interference by quinols such as pyrogallol and hydroquinone.

2. The determination of a complete calibration curve and calibration constant need be carried out only occasionally. One or two points on the curve may be checked every now and then. In our laboratories the value obtained for K has been very constant for a considerable period of time.

E. CONVERSION OF DEHYDROASCORBIC ACID TO REDUCED ASCORBIC ACID BY HYDROGEN SULFIDE TREATMENT

Reagents (other than those described under D).

Sodium Hydroxide Solution. Approximately 5%.

Alkaline Pyrogallol Solution. Approximately 25 g. NaOH and 5 g. pyrogallol in 250 ml. of water. Prepare fresh each time.

Caprylic Alcohol. C.P.

Lead Acetate Paper.

Cylinder of Hydrogen Sulfide Gas.

Cylinder of Nitrogen Gas.

Apparatus.

All-glass equipment is desirable for bubbling the gasses through the extracts. For the injection of H_2S, the gas train consists of $H_2S \rightarrow$

water (to saturate the gas with moisture) → extracts → NaOH (to absorb excess gas). For removal of the H_2S with nitrogen, the gas train consists of N_2 → alkaline pyrogallol (to free the gas of oxygen) → extracts → NaOH. The extracts are placed in 50 ml. amber cylinders with gas inlets and outlets, the other liquids in 250 ml. or 500 ml. gas washing bottles.

Procedure.

The same metaphosphoric acid extract is used for determining both the reduced and the dehydro forms of ascorbic acid. An aliquot of the extract, 3-4 times the volume of the aliquot taken for colorimetry as above, is placed in the 50 ml. amber cylinder, buffered to pH 3.5 with acetate buffer (1 ml. of buffer/5 ml. of extract) and diluted to 25 ml. with water. Two to 6 drops of caprylic alcohol are added to minimize foaming. A series of extracts—up to about 12—is connected in the gas train described in the section on apparatus. H_2S is allowed to bubble through at a moderate rate for 15-20 min. The H_2S cylinder valve is closed almost completely and the gas train is allowed to stand (away from direct sunlight) for about 2 hr. with a slight positive gas pressure. Then wet nitrogen is passed through the train until most of the excess H_2S is removed (about 1 hr.). An aliquot (usually 5 ml.) of the treated extract is taken and an equal volume of 3% H_2O_2 added to remove any excess H_2S; no buffer is added, however, since the extract is already buffered at pH 3.5. Colorimetry and calculations are the same as for reduced ascorbic acid. The value obtained on H_2S-treated tubes represents total ascorbic acid. If the concentration of dehydroascorbic acid *per se* is desired, reduced ascorbic acid is determined and subtracted from this total.

F. DINITROPHENYLHYDRAZINE PROCEDURE FOR DEHYDROASCORBIC ACID AND TOTAL VITAMIN C

Reagents.

2,4-Dinitrophenylhydrazine. Dissolve 2 g. of 2,4-dinitrophenylhydrazine in 100 ml. of approximately 9 *N* H_2SO_4 (3 parts of H_2O to 1 part of conc. H_2SO_4) and filter.

Acid-Washed Norit. Place 200 g. of Norit in a large flask and add 1 l. of 10% HCl. Heat to boiling. Filter with suction. Remove the cake of Norit to a large beaker, add 1 l. of distilled water, stir up thoroughly and filter. Repeat this procedure, until the washings give a negative or very faint test for ferric ions. Dry overnight in an oven at 110-120° C. (Do not heat to high temperatures.) The washing of the Norit is recommended for complete protection against interference.

Mixed Acid. A solution containing 5% metaphosphoric acid and 10% acetic acid.

Sulfuric Acid (85%). To 100 ml. of distilled water add 900 ml. of conc. H_2SO_4 (sp.gr.1.84).

Thiourea Solution. Dissolve 10 g. of thiourea in 100 ml. of 50% (by volume) aqueous ethyl alcohol. This reagent is stable for at least 2 months.

Apparatus.

A *photoelectric colorimeter* with a filter absorbing at 540 mμ.

Procedure.

A weighed sample or suitable aliquot of a liquid containing 0.2-2 mg. of ascorbic acid is extracted with 50-70 ml. of mixed acid, made up to 100 ml. with mixed acid, and filtered. To 50 ml. of filtrate 1 g. of Norit is added, the mixture is shaken vigorously and filtered. Highly pigmented plant filtrates may require treatment with Norit 2 or 3 times for complete clarification.

Three to 5 ml. of Norit filtrate are placed in each of two matched photoelectric colorimeter tubes. One drop of 10% thiourea solution is added to each tube. One tube is used as a blank; to the other tube 1 ml. of the 2,4-dinitrophenylhydrazine reagent is added. The latter tube is placed in a constant temperature bath at 37° C. and kept immersed in the bath for exactly 3 hr. Then the tube is removed and placed in a beaker of ice water containing generous quantities of ice. The blank tube is also placed in the ice water. To each of these tubes, while in the ice water bath, 5 ml. of 85% H_2SO_4 are added very slowly, a drop at a time, from a burette; this step should require about 1 min. (The burette stopcock should be greased with H_2SO_4). It is important not to allow the temperature of the solution to rise appreciably in order to avoid positive errors due to charring of organic matter. Finally 1 ml. of the 2,4-dinitrophenylhydrazine reagent is added to the blank tube. Both tubes are shaken thoroughly under the ice water to obtain complete mixing and are then removed to a rack. After 30 min., the tubes are wiped dry and clean and read in a photoelectric colorimeter at 540 mμ.

A calibration curve is run with standard solutions of dehydroascorbic acid ranging from 0.25 to 15 γ/ml. To prepare the dehydroascorbic acid standard, a solution of 25 mg. of ascorbic acid in 25 ml. of 5% metaphosphoric acid is treated with 1-2 drops of bromine, shaken until yellow, decanted from the excess of bromine into a large test tube, and aerated until colorless. Standards of appropriate concentrations are made by diluting with the mixed acid (5% metaphosphoric and 10% acetic acids).

Notes on the Procedure.

1. The authors (323) obtained good agreement with the indophenol method on 32 plant tissues.

2. The method measures total vitamin C. If the concentrations of dehydroascorbic acid and ascorbic acid are desired, the Norit treatment is omitted. The sample is extracted directly in a solution containing 5% metaphosphoric acid and 1% thiourea, the mixture is filtered and an aliquot of this filtrate taken for colorimetry.

3. Recently, Bolin and Book (339) recommended the use of dichlorophenolindophenol in the place of Norit or bromine for the oxidation of ascorbic acid in the method of Roe and Oesterling (323). The excess dye is removed with thiourea. This method permits rapid determination of reduced and dehydroascorbic acid on the same acid extract by comparison with a single standard curve, provided that all determinations have the same final metaphosphoric acid concentration.

4. An application of the Roe and Kuether method (322) to the determination of the vitamin C content of blood serum has been made by Lowry, Lopez and Bessey (340). A micro modification of this method has recently been developed by Bessey, Lowry and Brock (341) for determining ascorbic acid in small amounts of white blood cells and platelets (p. 303).

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Microchemical Methods *

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* The methods described in this chapter were devised for the study and assessment of nutritional problems and nutritional status in general. As a consequence, methods for serum iron and serum protein are included with the vitamin methods.

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I. Introduction

The concentrations of many of the nutritive essentials or their derivatives (vitamins, minerals, proteins) in the blood, urine, other body fluids, and in tissues reflect, within certain limitations and under certain conditions, the recent dietary intake of these substances. This fact makes possible the use of chemical analytical methods as means of evaluating nutritive status. Elsewhere in this book a number of such methods have been described, which require for each analysis 1-5 ml. of blood or other body fluids.*

In this chapter, a number of micro methods will be described, in which only 0.03-0.05 ml. of serum, plasma, or cells are required for each analysis, thus making it possible to obtain enough blood by simple finger-tip puncture to test for a number of important dietary constituents. The convenience of the collection of blood specimens by this procedure, compared with that by the venous route, is considerable, particularly when studying large population groups, in cases when repeated sampling is necessary, or when working with infants and children. While successful analysis on a micro scale requires attention to details, the care required is no more than that necessary for satisfactory analysis on any other volume scale. The usefulness of the methods in studies involving small animals, and their adaptability for analyses of small tissue specimens, *e.g.*, biopsy specimens, is also important. Additional advantages are the rates and convenience with which the analyses can be performed in the laboratory.

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* See also Vol. II.

Since there are a number of techniques, such as collection of specimens, measurement of small volumes, microcolorimetry, *etc.*, which are common to a number of the micro methods, these will be described first. The methods described in this chapter are the product of the efforts of Otto A. Bessey, Oliver H. Lowry, Helen B. Burch, and a number of technical assistants. The reader should consult the original papers for more complete details of the methods (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11).

II. Collection of Specimens

1. Serum

A. MATERIALS

1. Blood-collecting tubes A (50 mm.^{3} capacity). Melting point capillaries, 1.5-2.0 mm. o.d., 7-10 cm. long (No. 34500, Kimble Glass Co., Vineland, N. J.).
2. Blood-collecting tubes B (300 mm.^{3} capacity). Pyrex tubing, 4.0 mm. o.d., 10 cm. long. These and the capillary tubes listed above and all other tubes used in the micromethods are cleaned by boiling in half concentrated HNO_3, rinsing in distilled water several times, boiling in distilled water, and finally rerinsing and drying in an oven at 45°-50° C. Chromic acid should never be used for cleaning.
3. Picene cement for sealing capillaries A (Pyseal, Eimer and Amend, New York; or Plicene, Central Scientific Co., Boston).
4. Sleeve stoppers (vaccine caps) for stoppering tubes B with outside sleeves cut off (No. 1A-66F, West Co., Phoenixville, Pa.).
5. Vacuum wax (11455-A, Central Scientific Co., Boston).
6. Bard-Parker blades (No. 11).

B. PROCEDURE

Blood is taken from the finger tip after washing with alcohol and lancing with a Bard-Parker blade held by projection from a small cork. Care should be taken to wipe off the first droplet of blood and all traces of alcohol before taking the sample. Squeezing the finger is permissible except when samples are being collected for hemoglobin determination. For volumes of serum up to 25 mm.^{3}, a capillary tube A is ¾ filled by capillarity. The tube is tipped until the blood runs to the middle, care being taken to keep one end of the tube dry. The ends are sealed as follows: The stick of picene is softened in a flame (match, candle or alcohol lamp) and applied to the dry end of the capillary which has also been warmed at the very tip in the side of the flame. The tight seal required at this end requires the glass to be warm enough for the picene to "wet" it. However, the blood must not be warmed or hemolysis will result. The other end of the capillary is plugged with a small piece of soft picene without warming the tube. The capillary is centrifuged 5-10 min. at 3,000 r.p.m. with the tightly sealed end down. A large number of samples may be centrifuged together by wrapping each in a small piece of paper bearing identification. A warm centrifuge should be avoided, or substances such as ascorbic acid may be lost.

By using 2 capillaries, volumes of serum up to 50 mm.^{3} are conveniently collected as described above. Volumes up to 150 mm.^{3} (300 mm.^{3} blood) are, however, more conveniently collected by the use of tubes B. By squeezing and, if necessary, by warming the hands, this amount of blood is easily and quickly obtainable. The first few mm. of the outside of the dry end of the tube are coated with vacuum wax or Vaseline and after filling, a vaccine cap is firmly pressed into place. The unwaxed end of the tube is closed with another cap. Before centrifuging, the caps on the waxed ends of the tubes are taped on with a strip of adhesive tape. Tubes are easily identified by rolling on a strip of paper, with number exposed, made fast by scotch tape.

When several hours may elapse before preparation can be made for analysis, it is wise to keep the blood specimens at or near 0° C. Under these conditions there will be no significant loss of ascorbic acid in 24-48 hours. In the field, or where transportation is necessary, 1 gal. Thermos containers (No. 820, The American Thermos Bottle Co., Norwich, Conn.), equipped with racks made from coarse screen wire, below which a tray of ice cubes may be packed, are useful.

After centrifuging, the tubes are opened by scratching the tube above the serum and also just above the clot with a diamond point and breaking. The serum is readily available for removal and/or aliquoting with a constriction pipette (see below). Serum to be preserved by freezing is transferred to 6 × 50 mm. tubes which are stoppered tightly with the vaccine caps described above to avoid evaporation.

2. Red Blood Cells and Plasma

A. MATERIALS

1. Blood-collecting tubes A or B (Section II, *1, a*).
2. Vaccine bulb (West Co., Phoenixville, Pa.).
3. Spot plate.
4. Heparin (*e.g.*, Liquaemin, Roche-Organon, Inc., Nutley, N. J.).
5. Prepared blood-collecting tubes. Into tubes A are measured 1 mm.^{3} of heparin solution, 6 mm.^{3} into tubes B. The tubes are tilted to wet the walls and then air-dried.

B. PROCEDURE

After collecting the blood in the usual way (Section II, *1, b*), the rubber bulb is immediately fitted to the dry end of the tube and the contents delivered into the spot plate and back into the tube several times to obtain thorough mixing. The tube and contents are then sealed, labeled, centrifuged, opened, and the cells and plasma aliquoted as previously described for serum.

3. White Blood Cells

A. MATERIALS

1. Serological test tubes, 6 × 50 mm. (*e.g.*, No. 45060, Kimble).
2. Racks for holding tubes; may be made of 3/8 in. wire mesh. Special racks made of Vinylite or stainless steel, holding 55 tubes are convenient and available (Herman Ruf, 5023 192 St., Flushing, L. I., N. Y.).
3. Pipette for transferring blood, 5-6 mm. in diameter, with a short tip narrowed to 1 mm. o.d. and with the opening at least 0.5 mm. in diameter. A coarse constriction placed at 0.1-0.12 ml. volume aids in preventing the accidental sucking of blood too far into the pipette. This pipette, although uncalibrated, is similar in construction to the quantitative constriction pipettes (Section III, *1, b*). The inside is paraffined by heating the pipette, sucking up molten paraffin, blowing it out, and, while still blowing, cooling with water. This leaves a thin film of paraffin effective in delaying blood clotting without unduly constricting the tip.
4. Footed stirring rod made of glass or stainless steel wire. The shaft is 1-1.5 mm. in diameter and 75 mm. long; the foot is 2.5-3 mm. in diameter and is flattened.
5. Pasteur pipette of 1-1.5 ml. capacity with bent tip for transferring white cell suspension.

6. A device for removing supernatant from white blood cells. A small glass tube with a bent narrow tip not over 0.5 mm. o.d. is connected with a rubber tube, through a 20-50 ml. bottle, to an outlet for suction by mouth.

7. Potassium oxalate, 1.6%. This is preserved at 4° C. to prevent growth of microorganisms, and must be centrifuged just before use to remove possible traces of suspended material.

B. PROCEDURE

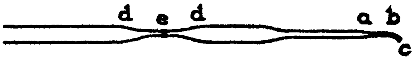
Into a 6 × 50 mm. tube are placed 0.5 ml. of 1.6% $K_2C_2O_4$ and a footed stirring rod. A finger is lanced to obtain a rapid flow of blood. After wiping off any residual alcohol, the area is lightly coated with Vasoline and the first blood blotted off. As rapidly as possible the paraffined pipette is filled, and the blood delivered into the tube, the contents of which are *at once* gently but thoroughly mixed with the rod. The pipette is rinsed with fresh oxalate but is not dried before again using. (The flow of blood from the finger must be free, and all operations must be conducted so as to avoid the slightest degree of clotting, since this will lead to white cells being carried down with the red blood cells.) The tube is centrifuged within an hour at a predetermined slow speed which settles the red cells without loss of too many white cells. A safe time of centrifuging is twice the interval required to just throw the *bulk* of the red cells to the bottom of the tube. The centrifuge must come smoothly to a stop to avoid remixing of the sample. The turbid supernatant, which contains the white cells, is transferred with a Pasteur pipette to another tube of the same size. The suspension close to the red cell layer should be avoided since this usually contains some red cells. The second tube is centrifuged (within 2 hr.) at 3,000 r.p.m. for 15 min. The clear supernatant is sucked off with the device described as item 6 above, and discarded. Great care is taken to remove a maximum amount of fluid without loss of the sediment. At first, magnifying glasses may prove helpful. If 90% of the fluid is removed and the tube allowed to stand 5 or 10 minutes for drainage, the last 10% can be removed more completely. The white cells are then ready for extraction as required.

III. Measurement of Small Volumes

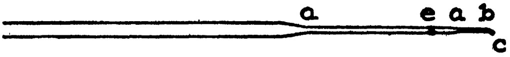
1. CONSTRICTION PIPETTES

A. PRINCIPLE AND USE

These pipettes were originally described by Levy (12) working in the laboratory of Dr. K. Linderstrøm-Lang. They are particularly useful for the easy, rapid, and precise measurement of volumes of 1-200 mm.[3] (0.001-0.2 ml.), although larger pipettes are occasionally of value. With 10 to 200 mm.[3] pipettes the percentage accuracy of delivery compares favorably with conventional pipettes delivering 2 to 10 ml., *i.e.*, approximately 0.1%. Below 10 mm.[3] the precision falls off somewhat, but even with 1 mm.[3] volumes a precision of at least 1% is obtained, and may be much better with a well-made pipette properly used. These pipettes are easy to construct for anyone familiar with the rudiments of glass blowing (see below), or they may be obtained from Microchemical Specialties Co., 1834 University Avenue, Berkeley 3, California.



Larger pipette (25-100 c.mm.)



Smaller pipette (1-10 c.mm.)

Fig. 1. Constriction pipette.

[Taken from O. A. Bessey, O. H. Lowry and M. J. Brock, *J. Biol. Chem.* 164, 321 (1946)]

Drawings of the constriction pipettes are shown in Fig. 1. The pipette is filled and emptied by the use of a small rubber tube such as is used with a blood-diluting pipette. The pipette is dipped not more than 1 or 2 mm. into the liquid, and, by sucking, liquid is pulled to just above the constriction. With gentle pressure the liquid level is blown down to the constriction, where surface tension stops the meniscus automatically. The pressure is not released until the tip of the pipette is removed from the remaining liquid. To deliver, the pressure is increased sufficiently to drive the meniscus past the constriction, and this pressure is maintained until devilery is complete (2-5 sec.). With smaller pipettes, the tip should be sufficiently constricted (by fire polishing) so that surface tension prevents air from following the liquid after it is delivered, since, otherwise, part of the liquid might be spattered. During delivery, the bend in the tip is used to keep the lower shaft of the pipette away from the wall of the tube into which the sample is being delivered. This is very important, since, otherwise, surface tension will cause the sample to run up the shaft of the pipette, and only part of the sample will be delivered into the tube, the rest clinging to the pipette. Similarly, the tip of the pipette must always touch a surface during delivery, since, if the tip is free in the air, much of the sample adheres to the outside of the pipette.

B. CONSTRUCTION

The following directions are for making an ordinary 50 $mm.^3$ constriction pipette. A 20-25 cm. length of either soft glass or Pyrex tubing, 4 or 5 mm. o.d., is heated in the middle and drawn down to a diameter of 1.5-2 mm. This furnishes material for 2 pipettes. Two cm. from that point where the tube is narrowed (Fig. 1, *a*) the slender portion is further drawn down to a diameter of 0.5-0.8 mm. This narrowest portion is bent 1 cm. below the 2 mm. portion (*b*) at an angle of about 45° from a straight line and is cut off with a diamond point 3 or 4 mm. below the bend (*c*). The upper large end is fire-polished. A 0.1 or 0.2 ml. graduated pipette is partially filled with water and placed horizontally on the table, a rubber tube is attached to the new pipette, its tip is touched to the tip of the graduated pipette, and 50 $mm.^3$ of water are sucked into the new pipette. A mark is made with the diamond point at the meniscus, and just above this point the pipette is narrowed, for a distance of about 1 cm. or so (*d-d*), to an *inner* diameter of perhaps 1.0-1.5 mm., without thinning the wall. Once again 50 $mm.^3$ of water are drawn in, and the meniscus should now fall in this narrow portion. If it does, a new mark is made with a diamond point and the actual constriction is made by heating just above this mark with as slender a flame as possible. Without pulling or pushing, the glass will thicken where the flame strikes it, and heating is continued until the bore is 0.1-0.2 mm. at its narrowed point (*e*). The constriction should be small enough to stop the meniscus from going by when moderate pressure is applied, but large enough so that undue pressure is not required to force the meniscus by. The opening at the tip (*c*) should be a trifle smaller than the upper constriction so that the pressure which pushes the meniscus past the upper constriction will not cause the pipette to deliver too rapidly. The delivery time for a 50 $mm.^3$ pipette should be 2-5 sec. With a little practice, pipettes within 1-2% of the desired volume can easily be made. However, each pipette is subsequently calibrated by delivery of water into a weighing bottle containing moisture, and weighing on a micro balance.

IV. Microcolorimetry

Colorimetry has been used widely as a means of analysis, primarily due to the inherent sensitivity of such methods, and to the simplicity of manipulation and instrumentation. Lowry and Bessey (4) have now devised a practical means for measuring the light absorption of very small volumes of fluids, thus making such methods even more sensitive and adaptable for the measurement of small quantities of many substances.

The Beckman spectrophotometer has been adapted easily for colorimetric measurements of volumes of the order of 60 $mm.^3$ (0.06 ml.) without sacrifice of length of light path through the specimen, and thus without loss of precision compared with the usual volumes. A narrow beam of light, formed by a special diaphragm, is allowed to transverse a narrow column of the specimen contained in a special narrow cuvette, and light absorption is measured in the usual way.

A. MATERIALS

1. Beckman spectrophotometer (Model D or DU).
2. Cuvettes (quartz); square cells with an i.d. of 2.4×10 mm. (for 60 $mm.^3$ volume) instead of the usual 10×10 mm., and 25 mm. high. Except for height, the outer dimensions are the same as those of the standard cells. The light path is 10 mm., the same as for the regular cuvettes. Cells of variable width for volumes of 36 to 150 $mm.^3$ have also been used.
3. Diaphragm attachment. A metal strip with 3 pinholes of various sizes drilled near one end, which slides into a channel cut into a sheet of metal which is inserted in the instrument between the spectral source and the cuvette carriage. Provision is made for the lateral adjustment of the position of the pinholes.
4. Cuvette holder; similar to the regular holder but provided with means for vertical adjustment of cuvette position.

All of the above equipment may be obtained from the Pyrocell Mfg. Co., 206 E. 84 St., New York 28, N. Y.

B. PROCEDURE

The metal holder for the diaphragm strip is inserted in the spectrophotometer between the cuvette carriage assembly and the body of the instrument by removing the bolts from the phototube housing. The sheet of metal is held in exact position by the holes drilled through it to match the precision pins of the instrument.

The pinhole to be used is adjusted laterally to give a maximum response to the photocells. The cuvettes are then placed in the special holder (or on small adjustable blocks which fit into the regular holder) and, with the photocell compartment removed and the carriage holder held in place, adjusted laterally by means of the carriage bolt, and vertically by means of the cuvette holder or blocks to *coincide* with the diaphragm. The photocell compartment is then replaced and the position of the pinhole adjusted laterally to give maximum response to the photocell through the cuvettes. These adjustments once made for cuvettes which are kept in their positions in the holder need only be checked from time to time. Provision is made so that either of 2 or 3 different-sized pinholes may be brought into position without disturbing the adjustment. With the diaphragm strip removed, the instrument may be used with standard cuvettes without disturbing the metal sheet.

Since there is usually a little "play" in the cuvette carriage, the cells should always be brought toward their position in front of the light from the same direction. If there are any traces of cement remaining on the cuvette walls, these should be removed with a needle, *etc.* The micro cuvettes are used like the larger cells except that, for convenience in handling, they are left mounted in the carriage. Samples are introduced with fine-tipped pipettes into the bottom of the cuvette, and the previous samples are removed by suction with a fine-tipped glass tube attached to a suction pump or aspirator bottle. If the glass tip is slender, 99% of the previous sample may be removed, thus eliminating the need for rinsing the cuvettes between analyses of a series, unless the concentrations are widely different.

The 2.4 mm. cells require about 60 mm.^{3} of solution. The exact amount necessary should be determined by pipetting in solution until further addition is without effect. A large cell may be used in the first position in the holder, which is merely for adjusting the instrument to zero for the particular solvent or other blank being used. The small cells should give blank readings that differ by no more than about 0.01 on the density scale from the first (large) cell, and these blank readings should be reproducible to within 0.002 on the density scale.

Colorimetric measurements on volumes of the order of magnitude described above are as rapid and convenient, and very nearly as precise, as with larger volumes (4).

V. Vitamin A and Carotene

1. SERUM

The usual Carr-Price (antimony trichloride) reaction (20) for vitamin A is not suitable for adaption to small volume measurements. The volatility of the solvent used, petroleum ether and chloroform, make manipulations almost impossible, and even slight evaporation leads to condensation of moisture and turbidity of the reagent. The evanescent nature of the blue color, and the difficulty of cleaning cuvettes, are other practical difficulties of the method.

Bessey, Lowry and Brock (7) have made use of a destructive irradiation technique to develop a method for measuring vitamin A and carotene in 60 mm.^{3} (0.06 ml.) of serum. Even smaller volumes (35 mm.^{3}) can be used, if particular attention is paid to technical details. The method is based on the further development of an observation made by Little (21) and others (22), that vitamin A under certain conditions can be selectively destroyed by irradiation with ultra-violet light.

Briefly, the method depends on (a) saponification and extraction of the vitamin A and carotene from serum on a micro scale with solvents of low volatility; (b) measurement of the light absorption of the small volumes at 328 mμ and 460 mμ; (c) destruction of the vitamin A spectral absorption at 328 mμ without affecting the absorption of other compounds at this wave length; and (d) remeasurement of the absorption at 328 mμ.

A. MATERIALS

1. 1 *N* KOH in 90% ethyl alcohol (1 volume of 11 *N* KOH + 10 volumes of absolute alcohol). The reagent should be prepared the day it is used. If a brown color develops rapidly, or if the reagent gives a blank, the alcohol should be refluxed with KOH and redistilled before use.

2. Kerosene-xylene mixture (1:1). Xylene, C.P., and odorless (water-white) reagent kerosene (obtainable from Eimer and Amend, New York. Some other types of odorless kerosene have been found to be unsatisfactory.)

3. Test tubes 10 cm. × 3 mm. Twenty cm. lengths of tubing, 3.0-3.5 mm. i.d., are cleaned by boiling in half-concentrated HNO_3, rinsed, dried, and divided in the middle with a hot, narrow, blast-lamp flame to yield 2 tubes ready for use. Pyrex tubes have been used but, presumably, soft glass would be satisfactory and easier to seal in the flame. It has been found easier to make new tubes than to clean old ones, since, after one end is sealed, cleaning is somewhat laborious owing to the narrow bore.

4. Soft glass tubes similar to those described above, but only 4 cm. long and 2.5-3.0 mm. i.d. These are made and cleaned in the same fashion as described above.

5. Constriction pipettes, 60 mm.^{3} (Section III, *1, b*). It is desirable to have the upper constriction quite small to permit pipetting of organic solvents with low surface tension. If the tip is slender and the bend in the end very short, it will facilitate the measurement of samples into the long narrow tubes.

6. B-H4 mercury discharge lamp with purple envelope and with its special transformer (General Electric Co.).

7. Arrangement for irradiating samples in the soft glass tubes (Fig.

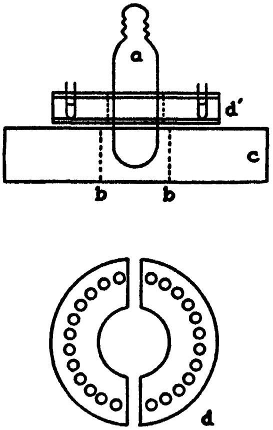


Fig. 2. Arrangement for ultraviolet irradiation.

The mercury lamp (*a*) is held vertically in a clamp, base up, with the other end extending 3 or 4 cm. into a hole 8 cm. in diameter, *b-b*, in a large block of wood, *c*, which serves as a base. Semicircular racks (*d*, *d'*) are provided for holding the glass tubes in a circle equidistant from the lamp (6 cm. from the center of the lamp). These racks may be made from pieces of ¼ in. plywood held about 2 cm. apart, with the upper piece drilled to hold the tubes. Twenty or 30 holes may be drilled in each rack along a semicircular line. [Taken from O. A. Bessey, O. H. Lowry, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166**, 177 (1946)]

2). When the racks are in position around the lamp, the brightest part of the light source should be opposite the lower halves of the tubes so that these portions of the tubes receive full illumination. The shadow of the electrode support must not fall on any tube. A moderate air current from a fan must be used to keep the tubes cool.

8. A Beckman spectrophotometer fitted with a micro attachment and quartz cuvettes, as previously described (Section IV, *a*).

9. Racks about 5 × 5 × 2 in. to hold 50-100 long tubes. These may be made from ½ in. mesh wire screen. Special racks of Vinylite or stainless steel, 3.5 × 7.5 × 2 in., holding 55 tubes, are convenient and available (Herman Ruf, 5023 192 St., Flushing, L. I., N. Y.)

10. Mixing device. The head of an eightpenny nail cut off, flattened for a distance of 10 or 15 mm. and inserted in a small high speed hand drill (*e.g.*, Handee grinder, Chicago Wheel Mfg. Co.) with the flat end projecting about 20 mm.

B. PROCEDURE

Into the long slender tubes are put 60 mm.^{3} of serum and 60 mm.^{3} of alcoholic KOH. If the solutions do not run to the bottom, they are sent down by a whipping motion. Mixing is accomplished by touching the side of the tube near the bottom to the whirling nail in the motor drill which has been mounted in a clamp with the nail up. The tube is immersed (along with others in a rack) in a water bath at 60° C. for 20 minutes, cooled, and 60 $mm.^3$ of kerosene-xylene mixture are added. Extraction is accomplished by holding the tube at about a 45° angle against the whirling nail in such a manner that the contents are violently agitated for 10 or 15 seconds. The tubes are then centrifuged 10 minutes at 3,000 r.p.m. They should be at room temperature, or a little below, before they are centrifuged. Each tube is cut with a file just above the kerosene-xylene layer and this layer is pipetted into the special narrow Beckman cuvette. All the solution possible is used, but with great care to avoid any of the aqueous layer, which would cause turbidity. The pipetting is best accomplished with a fine-tipped constriction pipette of about 50-60 $mm.^3$ volume. However, this pipette need not be calibrated. The constriction acts merely as a brake to prevent the sample from being accidentally drawn up too far in the pipette.

Readings are made at 460 and 328 mμ. The sample is then removed to a short soft glass tube (item 4 above) and irradiated, along with the other samples, with the B-H4 lamp. The lamp must have been turned on at least 10 minutes prior to the beginning of the irradiation. The necessary irradiation time (30-60 min.) should be determined by trial with known vitamin A solutions. Irradiation is applied for 6 or 8 times, as long as is found to be necessary to destroy 50% of the vitamin A in pure solutions. To eliminate the danger of a turbidity of unestablished origin, which sometimes develops, and which may be so slight as to be unnoticeable and yet serious enough to cause real error, the pipette used to transfer the sample back into the cuvette after the irradiation is rinsed before each sample with anhydrous propionic acid for one-third to one-half its length below the constriction. This procedure neither adds significantly to the analytical time nor changes the volume enough to cause error. If necessary or desired, the propionic acid may be added prior to the first reading, since its incorporation does not affect the course of irradiation. A substitute for propionic acid, which may possibly be more satisfactory, is a 1:1 mixture of xylene and 2-methyl-2,4-pentanediol. The addition of a great excess of propionic acid may, in some cases, itself induce turbidity, whereas pentanediol appears to be miscible with serum extracts in all proportions. The xylene is added to reduce viscosity.

C. CALCULATION

$E_{460} \times 480 = \gamma\text{-}\%$ carotene

$E_{328} - E_{328}$ irr. $\times\ 637 = \gamma\text{-}\%$ vitamin A (alcohol)

E = optical density with a cell having a 1 cm. light path $= 2 - \log \%$ transmission with such a cell. Optical density is read directly on the Beckman spectrophotometer. If the volumes of serum used are not equal to the volumes of kerosene-xylene used, these factors must be multiplied by $\frac{\text{Volume kerosene-xylene.}}{\text{Volume serum}}$

The factor of 637 for vitamin A is based on an $E_{1\text{ cm.}}^{1\%}$ for vitamin A palmitate in alcohol of 1720 at 328mμ, calculated as free alcohol (23). The extinction coefficient of the vitamin A ester was used for the following calculation rather than that of the free alcohol because most of the vitamin A in serum is esterified and saponification is quite incomplete. Since vitamin A ester has only 96% as much absorption in kerosene-xylene and still has 3% of its initial absorption left after irradiation, and since, furthermore, the absorption is reduced 2% owing to the necessity of using a wide spectral band (8mμ), the net $E_{1\text{ cm.}}^{1\%} = 1720 \times 0.96 \times 0.97 \times 0.98 = 1570$, and $\frac{1{,}000{,}000}{1570} = 637$.

The factor of 480 for carotene was obtained by measuring the absorption of β-carotene (Smaco) in kerosene-xylene ($E_{1\text{ cm.}}^{1\%} = 2080$).

If desired, the volume of serum and reagents may be increased or decreased proportionately. The ratio of serum to alcohol must be kept constant, but the amount of kerosene-xylene may be varied independently.

D. DISCUSSION

Saponification and extraction are conveniently carried out in the long slender tubes, which prevent undue evaporation during saponification and which give sufficient fluid depth to facilitate subsequent removal of the organic solvent layer. Saponification presents no problem and it scarcely prolongs the analytical time, since it is as easy to add alcoholic KOH as it is to add alcohol alone, which must be added in any event, and as many as 100 samples may be saponified at once in a single water bath. The saponification is not complete, glycerides are only partially hydrolyzed, and the same is probably true for vitamin A esters. However, the alkaline treatment accomplishes its purpose, *viz.*, facilitation of vitamin A extraction and the removal of interfering materials (7).

If a mixing device such as is described above is not available, mixing and extracting may be accomplished by adding a 1 cm. length of 0.041

in. diameter stainless steel wire (Newark Wire Cloth Co., Newark, N.J.) and shaking. Mild agitation suffices to mix the alcoholic KOH with the serum, and after adding the kerosene-xylene, the tubes are sealed at the upper end in a flame and shaken vigorously. Up to 50 tubes may be shaken together by hand (200 or 300 times). For a large series of analyses this technique is as rapid as the one given above; however, it is necessary to take great care to prevent any serum from wetting the top of the tube, which would result in charring when the tubes are sealed off, and thereby jeopardize the analysis.

A major problem was to find a solvent which would completely extract vitamin A and carotene from serum and which would permit manipulation of small volumes without undue evaporation. Petroleum ether was completely unsuited owing to its volatility; toluene was an improvement; xylene was still better; and kerosene showed practically no evaporation but failed to extract vitamin A or carotene quantitatively. A 1:1 mixture of kerosene and xylene was found to have such a low volatility that evaporation could be ignored, and recovery experiments from serum demonstrated the quantitative extraction of both pigments (7).

Measurement of light absorption in small volumes with the Beckman spectrophotometer has been previously described (Section IV, *b*). However, since, in this case, the samples are transferred after first readings from the absorption cuvettes to small tubes for irradiation, and transferred back to the cuvettes for the second readings, there is danger that so much of the sample might be lost during the manipulations as to leave an insufficient volume for the second readings. Such loss can be prevented by using slender-tipped transfer pipettes and making sure that no more than a trace of liquid is left in either cuvette, irradiation tube, or transfer pipette.

E. REPRODUCIBILITY AND COMPARISON WITH CARR-PRICE METHOD

Replicate analyses of 25 sera by this method gave standard deviations of 1-2 γ-% for vitamin A and 1 γ-% for carotene. Comparisons of the method with Carr-Price determinations on saponified extract of 11 sera gave standard deviations between individual values measured both ways of 5 γ-% for vitamin A and 7 γ-% for carotene. Evidence for the selectivity of the ultraviolet irradiation in the destruction of vitamin A may be found elsewhere (7).

F. STABILITY

In making analyses for nutritional surveys it is most convenient if samples can be collected in the field, transported to a central laboratory, and analyzed at a later date. In this case one must be assured of the keeping quality of samples, and of the storage conditions compatible with the stability of the substances to be measured. To obtain the necessary information, 2 serum samples were stored at various temperatures in a number of sealed tubes and analyzed after 1 and after 4.5 months for vitamin A and carotene by the proposed micro method. There was no detectable change after 1 month at either 4° or − 20° C. in either vitamin A or carotene. At room temperature, however, the vitamin A had fallen to about 45% of its initial value and the carotene to 10% or less. After 4.5 months at − 20° C., there was no significant change in vitamin A and the carotene had only fallen by about 6%. At 4° C. the carotene was almost gone in both samples, one vitamin A value was unchanged, and the other had fallen 40%. It seems permissible to conclude that, in serum, carotene is more unstable than is vitamin A, and that sera may be stored for several weeks at 4° C., or several months at − 20° C., without prejudice to the results.

VI. Ascorbic Acid

1. SERUM

The Roe and Kuether dinitrophenylhydrazine method (13), which depends upon the quantitative development of a colored product when this reagent reacts with oxidized ascorbic acid (dehydroascorbic acid), has proven more adaptable to a microprocedure than the widely used indophenol method (14). The method is sensitive enough to permit satisfactory analysis with 10 mm.^{3} of serum, manipulations are simple and practical, and methods of preservation are more adequate and practical than for other methods. In adapting the method to small volume analysis, it was found necessary to make certain simplifications of procedure which should prove helpful in large volume work as well (3).

A. MATERIALS

1. Reagent. Two per cent 2,4-dinitrophenylhydrazine, 0.25% thiourea, 0.03% $CuSO_4.5H_2O$ in 9 *N* H_2SO_4. This reagent is stable for at least a week at 4° C., but should be centrifuged before use unless crystal-clear. It is prepared from the stable solutions of 2.2% 2,4-dinitrophenylhydrazine (Eastman Kodak Co., Rochester, N.Y.) in 10 *N* H_2SO_4 by the addition of 5 vol.-% of 5% thiourea and 5 vol.-% 0.6% $CuSO_4.5H_2O$. The H_2O solution of thiourea is satisfactory for use for 1 month if left at 4° C. The copper sulfate solution, which is prepared in 0.01 *N* H_2SO_4, is stable indefinitely.

2. H_2SO_4, 65% (70 ml. concentrated $H_2SO_4 + 30$ ml. H_2O).
3. Trichloroacetic acid, 5% aqueous solution.
4. Constriction pipettes (Section III, *1, b*), 10, 30 and 50 mm.^{3} size.
5. Beckman spectrophotometer fitted with special diaphragm and cuvettes to permit use of 0.06 ml. fluid volumes, as previously described (Section IV, *a*).
6. Serological tubes, 6 × 50 mm. (*e.g.*, No. 45060, Kimble).
7. Racks for tubes as previously described (Section V, *1, a*).

B. PROCEDURE

Ten mm.^{3} of serum are delivered just above the level of 40 mm.^{3} of 5% trichloracetic acid contained in a 6 × 50 mm. tube. The contents are mixed by tapping the tube with the finger, and the tube is capped with a rubber stopper and centrifuged 10 minutes at 3,000 r.p.m. A 30 mm.^{3} aliquot of supernatant is transferred to another 6 × 50 mm. tube, and 10 mm.^{3} of the copper sulfate-thiourea-dinitrophenylhydrazine reagent are added with tapping. The tube is capped as before, and incubated 4 hr. at 38° C. It is now chilled in water, 50 mm.^{3} of ice-cold 65% H_2SO_4 are added, and the mixture very thoroughly mixed with the mechanical device previously described (Section V, *1, a*). Blank and standards are provided by treating 30 mm.^{3} aliquots of 4% trichloroacetic acid and of fresh 0.2 mg.-% ascorbic acid in 4% trichloroacetic acid with the reagent, *etc.*, in the same manner as the serum extract.

C. CALCULATIONS

$$\text{Mg. ascorbic acid/100 ml.} = \frac{D_{AA}}{D_{Std.AA}} \times \text{conc. std. (mg.-\%)}\ \frac{50}{30}$$

Where D_{AA} and $D_{Std.AA}$ are the optical densities of unknown and standard, respectively, corrected for the blank and where $\frac{50}{30}$ is a correction for the aliquot taken for analysis.

D. PROPORTIONALITY, REPRODUCIBILITY AND COMPARISON WITH OTHER METHODS

The color development was found to be satisfactorily proportional on the micro scale. For example, with standard solutions equivalent to 0.2, 0.4, 0.8, 1.6, and 3.2 mg.-% of serum, observed density readings were 0.013, 0.034, 0.069, 0.133, and 0.276, whereas the readings, if proportional, should have been 0.017, 0.034, 0.069, 0.138, and 0.276. Replicate determinations agree satisfactorily. For a series of 46 sera ranging from 0.3 to 1.4 mg.-%, analyzed in duplicate, the estimated standard deviations of the individual determinations were 0.03 mg.-%. (Estimated standard deviation = $\frac{\sqrt{\Sigma \Delta 2},}{2 \times n}$ where Δ = difference between duplicate analyses and n = number of sera analyzed. This statistic is equivalent to the standard deviation that would be obtained if n replicate analyses were done on a single serum).

Analyses of 9 sera, by both the indophenol method of Mindlin and Butler (14) and the nitrophenylhydrazine method described above, indicate that the latter method gives values that, on the average, are about 0.1 mg./100 ml. higher.

The method as described is readily adapted to larger volumes of serum, *i.e.*, 25, 50 mm.^{3}, *etc.* by simply increasing all volumes proportionally.

E. STABILITY

Once the acid has been added to the serum, it may be safely stored several days in the icebox, or for weeks at − 20° C. without change in the ascorbic acid content. One must, however, guard against evaporation. The small vial stoppers recommended for closing the blood-collecting tubes are most effective for this purpose. Serum left in the clotted blood in the collection tubes is stable for several hours, up to 24-48 hours if cooled (4° C.). Separated serum may be left for 2-4 hours at room temperature without serious loss. However, serum at 38° C. will show detectable lowering of the ascorbic acid analysis in 1 hour. Care should be taken to cool samples on warm days or while in the centrifuge. The advantage of being able to store acidified samples until a large number have been collected is obvious. Because of this, and the other advantages ascribed to it, the proposed method appears to satisfy the requirements of a micro method for purposes of nutritional surveys.

2. White Blood Cells

A knowledge of the concentration of ascorbic acid in the white blood cells and blood platelets has been shown to be of value as an addition to the blood plasma analysis in evaluating nutritional status, particularly at low levels of ascorbic acid intake (15, 16, 17, 18, 19). Furthermore, as it is probably more convenient to obtain specimens of white blood cells than of any other living cells of the body, it seems likely that analysis of these cells will become of increasing value in studying the physiology of ascorbic acid and other nutritive essentials in body fluids and tissues.

By a procedure previously described (Section II, *3, b*) it has proven feasible to isolate the white cells from as little as 0.1 ml. of blood. Described below is a method for analyzing this small amount of cells for ascorbic acid (8). The difficulties of accurately measuring such small quantities of material have been avoided by referring the analysis to the acid-insoluble phosphorus content of the cells instead of to weight. The measurement of this nearly constant phosphorus fraction by a microcolorimetric method permits calculation of the amount of white cells present in the sample. The ascorbic acid content of the white cell extract is determined by the same procedure used for serum.

A. MATERIALS

1. Reagents and equipment (Section VI, *1, a*).
2. 4.5 *N* H_2SO_4.
3. Perchloric acid, 70%.
4. Phosphate reagent. In 45 ml. of water is dissolved 0.3 g. of a powder consisting of 5% Na_2SO_3, 94.3% $NaHSO_3$, and 0.7% 1,4-aminonaphtholsulfonic acid. To this is added 5 ml. of 2.5% ammonium molybdate in water.

B. PROCEDURE

The white cells, obtained as previously described (Section II, *3, b*), are distributed as thinly as possible over the bottom of the 6 × 50 mm. tubes by tapping, and 40 $mm.^3$ of 5% trichloroacetic acid are added. For adequate extraction of ascorbic acid and acid-soluble phosphorus, it is imperative that the precipitate be well dispersed. This is best accomplished by use of the mechanical mixing device previously described (Section V, *1, a*). After centrifuging, a 30 $mm.^3$ aliquot of the supernatant is transferred to another 6 × 50 mm. tube and the ascorbic acid analysis conducted as described for the serum. (The residue is saved for measuring the acid-insoluble phosphorus (see below).) Blanks and standards are provided by treating 30 $mm.^3$ aliquots of 5% trichloroacetic acid and of fresh 0.4% ascorbic acid (0.004 $\gamma/mm.^3$) in 5% trichloroacetic acid in the same manner as the extract.

The ascorbic acid analysis may be deferred indefinitely if desired by storing the 30 $mm.^3$ aliquots in well-stoppered tubes at − 20° C. or colder.

C. ACID-INSOLUBLE PHOSPHORUS ANALYSIS

After removing the aliquot for ascorbic acid analysis, the residue is washed (within 1 hour at room temperature or 4 hours at 4° C.) by mixing with 0.2-0.3 ml. of 5% trichloroacetic acid, centrifuging, and sucking off the supernatant. Thirty $mm.^3$ 4.5 *N* H_2SO_4 are then added, and the sample (with others in a rack) is heated for 1 or 2 hours in an oven at 95-98° C. to drive off excess moisture without spattering, after which ashing is accomplished by adding 10 mm.^{3} of 70% perchloric acid and heating in an oven for 2 hours at 145-160° C.

Color is developed by adding 0.2 ml. of the phosphate reagent. The samples are vigorously mixed and are mixed again by tapping with the finger just before reading.

Blanks are supplied by measuring into 6 × 50 mm. tubes 30 mm.^{3} of 4.5 N H_2SO_4. Standards are provided by substituting an equal volume of 0.5 mM KH_2PO_4 in 4.5 N H_2SO_4 (0.015 μM P). These blanks and standards are carried through the same evaporation and ashing procedures as the white cell samples. The optical absorption is measured as 690 mμ not less than 20 minutes after mixing. Subsequent color development is slow and occurs at a uniform rate. Some of the standards are read both before and after the unknowns to permit correction for any increase in optical density with time.

D. CALCULATION

The white cells have been found to contain an average of 3.34 mM acid-insoluble phosphorus/100 g. (8). Thus, the mg. of ascorbic acid /3.34 mM acid-insoluble phosphorus (or $\gamma/3.34\mu M$) are numerically equal to the concentration/100 g. of white cells. Therefore,

$$(1)\quad \frac{\gamma \text{ ascorbic acid in sample}}{\mu M \text{ P in sample}} \times 3.34 = \frac{\text{mg. ascorbic acid/100 g.}}{\text{white cells}}$$

$$(2)\quad \gamma \text{ ascorbic acid in sample} = 0.004 \times \frac{D_{AA}}{D_{Std.AA}} \times 41.5 = K_1 \times D_{AA}$$

Where D_{AA} and $D_{Std.AA}$ are the optical densities of unknown and standard, respectively, corrected for the blank, and where 41.5 is the sum of the 40 mm.^{3} acid added + approximately 1.5 mm.^{3} of fluid left behind with the original white-cell sediment, and 0.004 equals the γ of ascorbic acid/mm.^{3} of standard.

$$(3)\quad \mu M \text{ P in sample} = \mu M \text{ P in standard} \times \frac{D_P}{D_{Std.P}} = K_2 \times D_P$$

In a series of analyses, the standards and their respective optical densities are fixed and, hence Eq. 1, 2 and 3 may be combined:

$$(4)\quad \frac{K_1 D_{AA}}{K_2 D_P} \times 3.34 = K_3 \frac{D_{AA}}{D_P} = \text{mg. ascorbic acid/100 g. white cells}$$

(The volumes of all the pipettes, except the 40 and 30 mm.^{3} cancel out, as they are used for both standards and unknowns.)

E. REPRODUCIBILITY AND COMPARISON WITH MACROMETHOD

The standard deviation of individual micro analyses was found to be 2 mg.-% in a series of 35 determinations on 9 different individuals. This is a coefficient of variation of about 7%. A comparison of 10 white-cell samples isolated from different bloods and analyzed both on a macro scale and a micro scale showed a standard deviation between the 2 methods of 3.6 mg.-% (coefficient variation of 12%). The macro samples were obtained by the procedure of Butler and Cushman (15) in which the cells are weighed. The micro samples and analyses were obtained by the method described above. Ascorbic acid was determined in both cases by the dinitrophenylhydrazine method.

VII. Riboflavin

1. SERUM, RED BLOOD CELLS, WHITE BLOOD CELLS

The concentrations of many of the dietary essentials in body tissues and fluids reflect the relatively recent dietary intakes of these substances. This fact has led to the usefulness of analytical methods for the evaluation of nutritional status. However, there has been a paucity of information on the relation of the riboflavin content of the blood to its dietary intake, primarily due to the lack of a satisfactory analytical method. The recent development by Lowry (10) of a very sensitive fluorometer now permits the measurement of riboflavin in concentrations of 0.2-0.5 mγ/ml. on small volumes (0.5 ml.) with a precision of about 3-5%. Methods developed by Burch, Bessey and Lowry (11) are described below which require but 20-25 mm.^{3} of serum, red blood cells, or whole blood, or the white blood cells from 0.1 ml. of blood, for a satisfactory determination of total riboflavin (11). An extra 25 mm.^{3} of serum also make possible a measurement of the riboflavin present in a free form in this phase of the blood (about 30% of the total). The riboflavin in the cells is almost completely in the form of the dinucleotide. Blood from normally nourished individuals shows a total riboflavin content for serum of about 3.5-4.0 γ-%, red blood cells 25 γ-%, and white blood cells 250-260 γ-%.

A. PRINCIPLE OF METHOD

Riboflavin (free and combined) may be completely extracted from serum, red blood cells, white blood cells, or whole blood, under appropriate conditions without splitting the combined form, flavin adenine dinucleotide (FAD). Measurement at pH 6.6-6.8 before and after reduction with hydrosulfite includes the fluorescence of any free riboflavin present plus 14% of the potential fluorescence which will arise when the FAD is split. Therefore, an additional measurement of the fluorescence, after splitting of the FAD in a second aliquot by acid hydrolysis, permits a determination of both the free riboflavin and the FAD riboflavin. The total riboflavin content of serum is the sum of these 2 forms. Since the riboflavin is almost completely in the form of FAD in the red cells and white cells, and since the fluorescence of the flavin mononucleotide (FMN) which arises due to the acid hydrolysis, has the same fluorescence as the free riboflavin, the total riboflavin and FAD riboflavin in these phases are the same and determinable by simple measurements of fluorescence after proper extraction and hydrolysis. A method is also available for the determination of the flavin mononucleotide, a combined form, which is present in important concentration in some tissue (11). However, this method is not described here, as it is of little importance in blood.

B. MATERIALS

1. Trichloroacetic acid, 10% aqueous solution.

2. Trichloroacetic acid, 5% aqueous solution.

3. K_2HPO_4, 4.0 *M* aqueous solution.

4. K_2HPO_4, 2.4 *M* aqueous solution.

5. K_2HPO_4, 0.16 *M* aqueous solution.

6. $Na_2S_2O_4$ (sodium hydrosulfite), 10% solution, in 5% $NaHCO_3$ (satisfactory for use only for a few hours).

7. Riboflavin standards. 0.2, 0.4, 0.8 and 1.6 γ/ml. in 0.01 *N* HCl made from a stock 20 γ/ml. solution. Standards are kept at 4° C., covered to protect from light. Aliquots taken for daily use are discarded. The protected standards are satisfactory for use for about a month.

8. Fluorescein secondary standards. Forty per cent alcoholic solutions equivalent to 1, 2, 3 and 4 γ/ml. riboflavin, kept stoppered in serological tubes; satisfactory for use for 2-3 months.

9. Reagents as described for phosphorus determination (Section VI, *2, a*).

10. Microphotofluorometer as described (10) or commercial instrument. (Farrand Optical Co., Bronx Boulevard and E. 238 Street, New York 6.)

11. Serological tubes, 6 × 50 mm.

12. Constriction pipettes 5, 20, 50, 100, 110, 400, and 500 $mm.^3$ (Section III, *1, b*).

13. Constriction pipette, 20 $mm.^3$ (calibrated to contain), wide tip for red cells.

Glass-distilled water is used for preparation of all reagents. All glassware is cleaned by boiling in 1:1 HNO_3 with thorough rinsing with distilled water. All determinations are done in a room illuminated only by red lights. Tubes and all glassware must be covered at all times to avoid contamination from dust, *etc.*

C. PROCEDURE (TOTAL AND FREE RIBOFLAVIN IN SERUM)

Fifty mm.^{3} of serum are added to 1 ml. of 5% trichloroacetic acid at 0° C. in a 3 ml. Pyrex test tube, mixed well, allowed to stand 15 minutes at 4° C., and centrifuged for 10 minutes in the cold. A 0.4 ml. aliquot of the supernatant is transferred at once to a fluorometer tube containing 0.1 ml. 2.4 *M* K_2HPO_4. A second 0.4 ml. aliquot is transferred to a dry fluorometer tube, and reserved for the determination of total riboflavin (see below). The apparent riboflavin content (A) of the tube containing the neutralized extract is measured in the microphotofluorometer within 1-2 hours of neutralization as follows: (meanwhile, care is taken to keep the tube in the dark.) By use of the secondary standards (fluorescein), the sensitivity of the instrument is adjusted so that the sample gives a reading (R_1) near the mid-point of the galvanometer scale. An internal standard (standard riboflavin solution) containing enough riboflavin in 5 mm.^{3} to give nearly a full scale deflection, is added, mixed and read (R_2). Five mm.^{3} of hydrosulfite reagent are added, mixed, and reading (R_3) made. Between each reading the instrument is rechecked with the secondary standard to be sure the sensitivity has not changed. All tubes must be carefully wiped before reading, to remove finger prints, *etc.* Measurements for correcting for the unavoidable fluorescent materials in reagents, *etc.*, are made by taking readings, before and after reduction with hydrosulfite, of a tube containing all reagents and the riboflavin standard. A reading on redistilled water is taken as a measure of scattered light and possible fluorescence of the tube itself. For measuring the total riboflavin of the filtrate the second 0.4 ml. aliquot is incubated (hydrolyzed) at 38° C. for 20 hours in the dark, after carefully stoppering with a paraffined cork or dust-free rubber stopper. It is then neutralized with 0.1 ml. 2.4 *M* K_2HPO_4 and its apparent riboflavin content (B) measured as described above.

D. CALCULATIONS

Corrections for volume changes due to reagent additions are made on the R_2 and R_3 readings and the resulting figures designated as R'_2 and R'_3. (The reading for distilled water is subtracted from the sample readings before correcting for dilution, since the contribution by scattered light would not be affected by dilution.)

The apparent riboflavin content of the aliquots A and B is calculated as follows:

$$\frac{\gamma \text{ riboflavin added}}{\text{ml. serum per aliquot}} \times \frac{R_1 - R'_3}{R'_2 - R_1} \times 100 = \gamma\text{-\% riboflavin}$$

The final figure is corrected by the value obtained for the appropriate blank determination.

Since, under the conditions of measurement, FAD before hydrolysis is 14% as fluorescent as riboflavin, then:

$$\text{FAD} = \frac{B - A}{0.86} \text{ and}$$
$$\text{Free riboflavin} = A - 0.14 \text{ FAD}$$

If a determination of total riboflavin only is desired, 25 mm.^{3} of serum are sufficient. The serum is precipitated with 0.5 ml. of 5% trichloroacetic acid at room temperature and may be allowed to stand 30 minutes to an hour before centrifuging. A 0.4 ml. aliquot is hydrolyzed and measured as above. Prolonged contact of the protein precipitate with the trichloroacetic acid should be avoided as being likely to increase the blank reading.

E. PROCEDURE FOR TOTAL RIBOFLAVIN IN RED BLOOD CELLS

Specimens of red blood cells are collected as previously described (Section III, *1, b*). The red blood cells remaining after removal of the white cell suspension (Section II, *3, b*) are also suitable for analysis. The small volume of supernatant remaining after the white cells are drawn off, together with the upper portion of the red cell layer, is discarded and the remaining red cells are packed by centrifuging at 4° C. for an hour. Any supernatant is removed.

A 20 mm.^{3} aliquot of the red cells is taken with a dry, rather wide-tipped constriction pipette. This is rinsed with 1.0 ml. of 1% NaCl solution. Three ml. of 13% trichloroacetic acid are added with mixing and allowed to stand for 30-60 minutes. After again mixing, the tube is centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant, which must be clear, is allowed to hydrolyze at 38° C. as described for serum. Aliquots of 0.4 ml. are pipetted into fluorometer tubes containing 0.1 ml. 4 *M* K_2HPO_4. The reducible fluorescence is measured as described above. The reduced reading must be made promptly after the addition of the hydrosulfite, or there is a tendency for the sample to reoxidize.

F. PROCEDURE FOR TOTAL RIBOFLAVIN IN WHITE BLOOD CELLS

The white blood cells from 0.1 ml. of blood, prepared as directed in Section II, *3, b*, are evenly distributed on the walls of the small tube and deproteinized by mixing thoroughly with 0.11 ml. of 5% trichloroacetic acid. The sample is allowed to stand at room temperature for 30 minutes to 1 hour and mixed again. After centrifuging at 3,000 r.p.m. for 10 minutes, 0.1 ml. of the supernatant fluid is transferred to a fluorometer tube and allowed to hydrolyze at 38° C. as previously described. The residue and remaining supernatant fluid are reserved for phosphorus determination. The hydrolyzed filtrate is mixed with 0.4 ml. of 0.16 M K_2HPO_4 and the total riboflavin measured as described for serum.

The acid-insoluble phosphorus is measured as described for white cell ascorbic acid, except for a simplification made possible because of the more dilute filtrate. Instead of washing the precipitate to remove acid-soluble phosphorus, the 10 mm.^{3} of acid solution which remain with the precipitate are included in the phosphorus analysis. To reduce the volume of fluid that needs to be evaporated, 20 mm.^{3} of 7 N H_2SO_4 may be conveniently substituted for the larger volume of 4.5 N acid previously recommended for digestion. Digestion of the sample, color development and measurement of the absorption at 690 mμ are carried out as previously described.

The sample analyzed for phosphorus contains all of the acid-insoluble phosphorus and, because of the 10 mm.^{3} of acid extract not removed, 9% of the acid-soluble phosphorus. Since white cells contain an average of 33 μM acid-insoluble phosphorus and 28 μM acid-soluble phosphorus/g. (8), the sample contains $33 + (0.09 \times 28) = 35.5$ μM/g. white cells in sample. Therefore, the γ of riboflavin/35.5 μM P found are numerically equal to γ of riboflavin/g. white cells, or

$$(1)\quad \gamma\text{-}\%\ \text{riboflavin} = \frac{\gamma\ \text{riboflavin in entire sample}}{\mu M\ \text{P found}} \times 100 \times 35.5$$

$$(2)\quad \gamma\ \text{riboflavin in entire sample} = \gamma\ \text{riboflavin found} \times \frac{110}{100},\ \text{therefore,}$$

$$(3)\quad \gamma\text{-}\%\ \text{riboflavin} = \frac{\gamma\ \text{riboflavin found}}{\mu M\ \text{P}} \times \frac{(110 \times 100 \times 35.5)}{(100\qquad)} =$$

$$\frac{\gamma\ \text{riboflavin found}}{\mu M\ \text{P found}} \times 3{,}900 = \frac{m\gamma\ \text{riboflavin found}}{\mu M\ \text{P}} \times 3.9$$

G. RECOVERY OF RIBOFLAVIN AND FAD

Riboflavin and FAD were added to serum, red and white blood cells prior to trichloroacetic acid precipitation. Filtrates, prepared in triplicate by the above-described procedures, showed average recoveries of 98-102% (11). Recoveries, however, were not complete with dilutions lower than those recommended, *viz.*, 1 : 20 for serum, 1 : 100 for white cells, and 1 : 200 for red blood cells. In fact, even at 1 : 200, recovery was found to be incomplete for red cells if 5% trichloroacetic acid was substituted for 10%.

H. REPRODUCIBILITY

Duplicate analyses of 13 sera for free riboflavin and for FAD gave an estimated standard deviation (Section VI, *1, d*) for the series of 0.07 γ-% for both the free riboflavin and FAD analyses.

In a series of 26 duplicate determinations of total riboflavin in serum, the estimated standard deviation was 0.08 γ-%.

The "average" standard deviation calculated from 3 to 5 analyses on each of 12 individuals was 11 γ-% for white cells and 1.3 γ-% for red cells.

VIII. Phosphatase

1. SERUM

A number of methods are in use for the determination of phosphatase (24, 25) in serum and other biological materials, all of which depend upon the principle of measuring the rate of hydrolysis, of glycerol phosphate, phenol phosphate or some other of a number of phosphate esters, under specified conditions of temperature, pH, *etc.* While these methods are quite satisfactory for many uses, they are unnecessarily time-consuming when large numbers of determinations are needed, and most of them require larger samples of serum than are conveniently obtained from a finger prick. A method has been developed recently (5) which obviates many of these difficulties. By the use of a new substrate (sodium *p*-nitrophenyl phosphate) a method has been devised which requires only 5 $mm.^3$ of serum (0.005 ml.), and which permits 50-100 analyses to be made in 2-3 hours. The simplicity and speed of the method recommends it for macro as well as micro determinations, and for either alkaline or acid phosphatase.

The reagent (substrate) sodium *p*-nitrophenyl phosphate is a colorless compound, but upon splitting off the phosphate group, the very chromogenic yellow salt of *p*-nitrophenol is liberated [absorption maximum 400 mμ (5)]. Hence, a product of the phosphatase reaction on the substrate serves directly as a measure of the enzymatic activity. Manipulations are simple; it is only necessary to incubate serum with the buffered reagent, stop the reaction by dilution with alkali, and measure the amount of color developed. Since serum itself makes a small contribution to the color, the first colorimetric reading is followed by the addition of acid to the sample (converting the yellow sodium salt into colorless free nitrophenol) and a second colorimetric reading which furnishes a blank correction. This procedure is considerably simpler than those now in use.

A. MATERIALS

1. Reagent A. Dissolve 7.50 g. (0.1 *M*) of glycine and 95 mg. (0.001 *M*) of $MgCl_2$ in 700-800 ml. of H_2O, and add 0.085 *M* (*e.g.*, 85 ml., 1 *N*) NaOH, and dilute to 1 liter.

2. Reagent B. Prepare 0.4% disodium *p*-nitrophenyl phosphate (Eastman Kodak Co., Rochester, N. Y.) in 0.001 *N* HCl. (At present the Eastman product contains about 50% inert material; hence, a double amount of this preparation should be used. If desired, the compound may be purified by recrystallization from hot 87% alcohol.) If the pH is not 6.5-8.0, adjust with acid or base. To test for free nitrophenol, dilute 1 ml. with 10 ml. of 0.02 *N* NaOH and measure the light absorption at 415 mμ. If the extinction is greater than 0.08 (*i.e.*, light transmission less than 83% for a 1 cm. light path or 70% for a 2 cm. light path), remove the free nitrophenol by extracting reagent B 2 or 3 times with equal volumes of water-saturated butyl alcohol, and once with water-saturated ether, finally aerating off traces of ether. Store in the icebox. Re-extract when reagent B fails to pass the above test.

3. Reagent C (complete reagent). Mix equal parts of reagents A and B. If necessary, adjust the pH to 10.3-10.4 with a little strong NaOH or HCl. Store in the refrigerator, or better, store frozen. When 2 ml. + 10 ml. 0.02 *N* NaOH have an extinction (1 cm.) greater than 0.1, either discard or extract with butyl alcohol and ether as above, and readjust the pH.

4. Standards. Prepare solutions containing 1, 2, 4, and 6 m*M*/l. of *p*-nitrophenol (Eastman Kodak Co.).

5. Constriction pipettes, 5 and 50 $mm.^3$ (Section III, *1, b*).

6. Serological tubes, 6 × 50 mm. (Kimble No. 45060).

7. A spectrophotometer or photoelectric colorimeter adapted to 0.5 ml. volume measurements, *e.g.*, Beckman spectrophotometer, or Coleman Junior spectrophotometer (Model 6). (Adapters for the Coleman instrument may be obtained from Samuel Ash, 1458 Seabury Place, New York).

8. Racks to hold tubes (Section II, *3, a*).

B. PROCEDURE

Serum samples (5 $mm.^3$) are transferred to the bottom of 6 × 50 mm. tubes held in a rack. A simple method for collecting small samples of serum has been previously described (Section II, *1, b*). The rack is immersed in a shallow pan of ice water, and to each tube are rapidly added 50 $mm.^3$ of ice-cold Reagent C, with a constriction pipette. Each tube is mixed by tapping with the finger. Care is taken not to warm the tube by so doing.

The rack of tubes is immersed in a water bath at 38° C. at a depth sufficient to cover the bottom half of the tubes. After exactly 30 minutes the rack is again placed in the pan of ice water and 0.5 ml. of 0.02 *N* NaOH are added to each tube with sufficient force to mix the sample. (A syringe pipette (Herman Ruf, 5023 192 Street, Flushing, Long Island, N. Y.) is very convenient for this purpose.) This addition stops the reaction and dilutes the samples, which are now transferred to colorimeter tubes and read at 400-420 mμ (R_1).

After the initial reading (R_1), 2-4 mm.^{3} of concentrated HCl are added with a 0.1 ml. graduated pipette (drawn-out tip) and a second reading (R_2) is made.

R_1 and R_2 are converted into optical densities ($-$ log transmission or 2 $-$ log per cent transmission) $=D_1$ and D_2. Then $D_1 - D_2 = D_c$, the corrected density.

Standards and blanks are provided by treating 5 mm.^{3} volumes of the standards and of distilled water exactly as though they were serum samples. The corrected densities (D_c) are used to construct a standard curve from which the serum values are calculated. Since sera and standards undergo the same dilution, it is unnecessary to take into account the exact volumes of the various pipettes. It is to be noted that D_c is the density corrected for possible residual absorption after acid addition but is not corrected for the D_c of the blank analysis. This second necessary correction is automatically provided by the standard curve. A "millimole unit" is defined as the phosphatase activity which will liberate 1 m*M* of nitrophenol/l. of serum/hr. Therefore, since the standard incubation time is only 30 minutes, the 1, 2, 4, and 6 m*M* standards are equivalent to sera with activities of 2, 4, 8, and 12 m*M* units. One such unit is approximately equal to 1.8 Bodansky units (see below) (5). For adult sera, which have low phosphatase activity, the volumes of serum and reagent may be doubled without increasing the volume of alkali; this will nearly double the amount of color.

C. PROCEDURE WITH 20 mm.^{3} OF SERUM

Volumes of serum, reagent C and 0.02 *N* NaOH are increased to 20 mm.^{3}, 200 mm.^{3}, and 2 ml., respectively. Otherwise, the procedure is nearly identical with that described for 5 mm.^{3} of serum. The sample may be conveniently incubated directly in 3/8 inch photocolorimeter tubes. One drop of 5 *N* HCl is added before the second reading.

D. PROCEDURE WITH 0.1 ML. OF SERUM

Use 0.1 ml. of serum, 1 ml. of reagent C, and 20 ml. of 0.02 *N* NaOH. Because of the larger volumes, the tubes containing 1 ml. of reagent are placed in the water bath and allowed to come to temperature before the addition of serum. As each serum sample is added, the time is noted and exactly 30 minutes later 20 ml. of NaOH are added.

E. DISCUSSION

The pH optimum for human serum has been found to be 10.0-10.1, and for rat serum pH 9.2-9.7, under the conditions described here (uncorrected glass electrode pH). Since alkaline phosphatase has a sharp pH optimum, deviations of more than 0.1 pH unit will affect the reading significantly. The reagent is well buffered so there is no danger of CO_2 from the air affecting the pH during incubation. However, the pH of the reagent should be rechecked occasionally (glass electrode) against a standard buffer of the same pH range, *i.e.*, 0.1 *M* sodium borate, pH 9.2.

Since time and temperature of incubation affect the enzyme activity, these conditions must be carefully standardized for reliable results. The degree of splitting has been found proportional to the concentration of phosphatase under conditions specified. However, beyond 30 minutes the rate may not be strictly linear with time. It is, therefore, desirable not to increase the period of incubation.

F. REPRODUCIBILITY AND COMPARISON WITH OTHER METHODS

The standard deviation of replicate determinations of the same serum by the proposed procedure with 5 $mm.^3$ has been found to be 0.15 m*M* units. Analyses of sera from 12 children by the Bodansky procedure (24) using 0.1 ml. of serum, and by the method described here using 5 $mm.^3$ (0.05 ml.) of serum, gave a ratio $\frac{(p\text{-nitrophenyl phosphate units})}{(\text{Bodansky units})}$ of 1.79, with a standard deviation of 0.10. Therefore, 1.79 Bodansky units (mg. of phosphorus liberated/100 ml. of serum/hr.) are equivalent to 1 *p*-nitrophenyl phosphate unit (m*M* of substrate hydrolyzed/l. of serum/hr.). One King-Armstrong unit (mg. of phenol split from phenyl phosphate/100 ml. of serum/hr.) is equivalent to 7.3 of the present units with a variance of 8%. (Personal communication, Dr. S. H. Jackson, Children's Hospital, Toronto, Canada).

G. STABILITY

The phosphatase content of serum does not change significantly at 4° C. for 48-72 hours, or at − 20° C. for 4 weeks.

IX. Iron

1. SERUM

Iron deficiency anemias are characterized by low serum iron, while other states of diminished hemoglobin formation show normal or even high values for serum iron. Therefore, in the absence of infection or other pathology, low serum iron is indicative of an iron deficiency (27, 28, 29, 30).

The prevalence of iron deficiency in population groups has not been widely investigated, due, in large part, to the lack of convenient methods. Traditional methods require 1-10 ml. of blood and time-consuming techniques. The procedure described below requires but 20 mm.^{3} of serum for a satisfactory iron analysis and as many as 75 such determinations can be done by 2 technicians in a day.

Briefly, acid and thiocyanate ion are added as one reagent to the serum and the iron (III) thiocyanate is extracted directly from the mixture with isoamyl alcohol. This avoids the preparation of serum filtrates and improves the extraction of iron from the serum proteins. A means is provided for correction for any error which might arise due to hemolysis of the blood specimen.

A. MATERIALS

1. All water is carefully redistilled from glass and stored in Pyrex glass-stoppered bottles.

2. NH_4SCN, 27%. Traces of iron are removed by adding 0.05 ml. concentrated HCl/100 ml. and extracting with a 3 : 1 mixture of isoamyl alcohol and ethyl ether until no pink color is visible. Stored at 4° C. this reagent is satisfactory for several months.

3. HCl, 1.35 *N*, prepared from redistilled 6 *N* (constant boiling) acid.

4. Isoamyl alcohol (redistilled) saturated with water and stored at 4° C. On the day used, to 10 ml. is added 0.1 ml. of 0.2% H_2O_2 prepared from 30% H_2O_2 (Superoxol).

5. Complete reagent. Equal volumes of 27% NH_4SCN and 1.35 *N* HCl are mixed for each series of determinations. This reagent keeps not more than 2 hours.

6. Standard iron solution. A stock 100 mg.-% iron solution is prepared from iron wire (Bureau of Standards) using redistilled HCl and a few drops of redistilled HNO_3. A working standard of 200 γ-% is prepared in 0.01 *N* HCl.

7. Ascorbic acid, 30% in ethylene glycol. Warming to 60° C. is necessary to dissolve completely. Higher temperatures result in discoloration. Preparation of fresh solution daily is recommended.

For 20 mm.³ of Serum.

8. Constriction pipettes 2, 20, 60 and 90 mm.³ (Section III, *1, b*) and 2 fine-tipped constriction pipettes of about 80 mm.³ capacity for transferring samples in and out of the spectrophotometer cuvettes.

9. Blood-collecting tubes (Section II, *1, a*).

10. Serological tubes 6 × 50 mm. (*e.g.*, Kimble No. 45060). These and all glassware used should be cleaned with boiling half-concentrated HNO_3 and thoroughly rinsed in distilled water.

11. Wooden or plastic racks for tubes (Section II, *3, a*).

12. Glass-stoppered flasks for reagents.

13. Centrifuge fitted with cups suitable for small tubes.

14. Beckman spectrophotometer adapted to small volumes. Micro cells of 2.0-2.4 mm. inner width are appropriate (Section IV, *a*).

15. Apparatus for agitating samples to effect the extraction of the aqueous phase with isoamyl alcohol (Section V, *1, a*).

For 0.150 ml. Serum.

16. Constriction pipettes, 0.015, 0.150, 0.45 and 0.6 ml., and 2 transfer pipettes of about 0.5 ml. capacity (Section III, *1, b*).

17. Pyrex test tubes, 5 ml.

Room should be free of unpainted iron ware and kept as free from dust as possible. All tubes, flasks, *etc.*, should be covered at all times.

B. PROCEDURE

Specimens of serum are obtained as previously described, except special care must be used that the finger is clean. It is, of course, necessary to work in rooms as free as possible from dust, and to take every precaution to avoid contamination of samples and clean vessels by covering whenever possible by dust-proof materials. To 20 mm.³ of serum in a 6 × 50 mm. tube are added 60 mm.³ of the complete reagent (Materials, item 5). The sample is well shaken at once by tapping to obtain a finely divided precipitate from which iron can be extracted. Ninety mm.³ of isoamyl alcohol containing H_2O_2 are added (Materials, item 4). The tube is shaken, covered with Parafilm, and mixed violently by vibration with the high speed motor and nail (Materials, item 15).

Blanks and standards are prepared at the same time by substituting either water or standard iron solution for the serum.

The tubes are chilled in centrifuge cups to 4-10° C. and then centrifuged at 3,000 r.p.m. for 10 minutes. Approximately 80 mm.³ of the upper isoamyl alcohol layer are pipetted into the Beckman cell with care to avoid contamination from the precipitate interface or from the water layer. (To clean the narrow Beckman cells, allow them to stand for 10-15 minutes filled with 1 *N* HCl, rinse well with redistilled water, and dry with alcohol and ether. Finally, one of the reagent blanks is used for rinsing the cells.) With isoamyl alcohol in the reference cell, the optical density of the sample is read at 520 mμ (R_1). The entire sample is removed from the cell with a fine pipette and delivered into a clean tube. Two mm.^{3} of the 30% ascorbic acid reagent are added and the solution is mixed. After 10-60 minutes, the optical density of the solution is again measured (R_2).

In practice, a series of samples are read, removed from the cells, and reduced with ascorbic acid. The tubes are covered with Parafilm and allowed to stand until the completion of the original readings on a series. Then the optical density of the reduced solutions is determined.

A method for correction for slight hemolysis is important, as it is extremely difficult to avoid this entirely. The correction is dependent upon the fact that while ascorbic acid completely reduces iron (III) thiocyanate, the light absorption of oxyhematin which may be present is not affected at the wave length used for measurements.

C. CALCULATIONS

Since the same pipettes are used for serum and for iron standard solutions, the volume of the pipettes does not enter the calculations.

$R_1 - R_2 = R_x$

$$\frac{(R_x - R_{\text{Blank}}) \times 200}{R_{\text{Std.}} - R_{\text{Blank}}} \times \frac{100}{90} = \gamma\text{-}\% \text{ Fe}$$

The factor $\frac{100}{90}$ is introduced to correct for the incomplete recovery (90%) of iron from serum (9). The concentration of reagents, method of extraction, *etc.*, have been adjusted to give the maximum reproducibility.

D. PROPORTIONALITY AND RECOVERY

The color produced has been shown to be proportional to the iron present under the conditions used for the analysis (9). Recoveries of iron added to serum varied between 87 and 91%, with an average of 90% (9). Serum, if protected from evaporation and contamination, may be stored at 4° C. or frozen indefinitely without change in iron analysis.

E. REPRODUCIBILITY

The standard deviation of triplicate analyses of blood taken from each of 4 fingers of 1 individual was 3.6 γ-%. Other replicate determinations confirm this order of precision for the method.

Hourly variations of serum iron of 20-30 γ-% are found in well-nourished individuals. Due to this fact, values above 60 γ-% cannot be interpreted other than as indicating an adequate iron supply. While values below 60 γ-% may also vary to some extent, experience has shown that it is reasonably safe to consider such levels as indicative of an inadequate iron supply (31).

X. Protein

1. SERUM

The specific gravity of serum has been shown to be a reliable index of serum protein concentration (32, 33). The falling-drop method of Barbour and Hamilton (34), the copper sulfate procedure of Philips *et al.* (35), and the gradient-tube method of Linderstrøm-Lang (36), are all suitable for measuring the specific gravity of serum. However, the simplicity, convenience, speed and accuracy of the gradient-tube method recommends it, not only for routine hospital use and experimental purposes, but it has been found almost ideally suited for large-scale population studies of protein nutrition. The facts that only 2 or 3 mm.^{3} of serum are required, and that determinations can be done by 2 persons at the rate of 40-50/hr., are particularly advantageous for survey work.

Although the use of the gradient tube for very precise specific gravity measurements had been carefully described (36), there had been but brief references to the adaptation of this method to the rapid measurement of serum or plasma until the paper by Lowry and Hunter (1) from which much of the following is taken.

A. PRINCIPLE OF METHOD

A column of organic liquid with a density which varies linearly from bottom to top can be established in a graduated cylinder by the proper mixing of a lower layer of a heavy liquid, such as bromobenzene, with a layer of a lighter liquid, such as kerosene. When droplets of serum, 2-4 mm.^{3} in size, are allowed to fall into this gradient, they come to rest at a point having a density exactly equal to their own. The density, and hence the protein concentration, are calculated graphically by interpolation from the position of rest of droplets of salt solution of known density. Such a gradient is satisfactory for many hundreds of measurements extending over a 6-10 months period. Temperature control is unnecessary. It is, however, helpful in preventing disturbances of the gradient by convection currents, to install the cylinder in a jar of water.

B. MATERIALS

1. Gradient tube and water jacket (Fig. 3). A 500 ml. graduated cylinder (A) fitted inside a larger cylinder (B). These cylinders may be obtained from Eimer and Amend, New York, or may be easily constructed in the laboratory from readily available material (37).

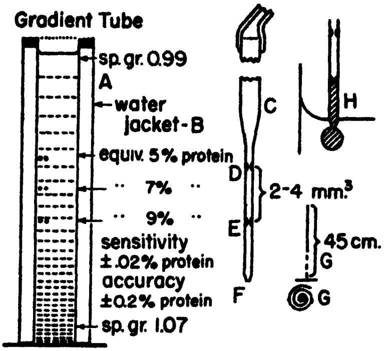


Fig. 3. Gradient tube for determination of serum protein concentration. A, graduated cylinder; B, water jacket; C, constriction pipette; D, E, and F, constrictions; G, wire stirrer; H, insertion of droplets. [Taken from O. H. Lowry and T. H. Hunter, *J. Biol. Chem.* **159**, 465 (1945)]

2. Gradient solution *A*. 100 ml. bromobenzene (sp. gr. 1.49), technical grade, + 150 ml. of kerosene (sp. gr. 0.80). White kerosene is preferable, although special purity is not required. If the specific gravity is not 1.07 ± 0.005 (urinometer), adjust to 1.07 with a little bromobenzene or kerosene.

3. Gradient solution *B*. 70 ml. of bromobenzene + 180 ml. of kerosene. Adjust, if necessary, to a specific gravity of 0.99 ± 0.005.

4. Jacket solution. 0.2% copper sulfate in 0.1% H_2SO_4 to fill the space between the 2 cylinders above. (The copper sulfate prevents molds and absorbs radiant heat.)

5. Constriction pipettes (Fig. 3, C), similar to those previously described (Section III, *1, b*) but adapted to deliver 2 droplets instead of 1. The constrictions differ in size, the upper one, D, being a little larger than the middle one, E, which is, in turn, larger than the bottom constriction, F.

6. Stirrer. A wire spiral made from heavy copper wire, 12-16 gauge (Fig. 3, G).

7. Blood-collecting tubes, A. (Section II, *1, a*).

8. Fine sea sand for removing droplets from the gradient tube.

9. Specific gravity standards. 250 g. of C.P. K_2SO_4 are dried overnight at 100-110° C. 17.64, 23.08, 28.53, 34.04, 39.58, and 45.10 g. amounts are weighed out (tolerance, 0.05 g.), and each sample is diluted to 1 liter to give 6 standard solutions of specific gravity, 1.0141, 1.0184, 1.0227, 1.0270, 1.0313, and 1.0356, which are equivalent to serum protein concentrations of 2.5, 4.0, 5.5, 7.0, 8.5, and 10.0%, respectively. These specific gravities are calculated *relative to water at the same temperature.* These standards will keep indefinitely if protected from mold and evaporation by storing in rubber-stoppered bottles in the icebox. Working standards are kept in 5 or 10 ml. rubber-stoppered bottles or tubes and are refilled from the primary standards every week or so.

C. PREPARATION OF GRADIENT TUBE

The graduated cylinder is filled to the middle graduation with solution *A*, and, on top of this, solution *B* is carefully layered to the upper graduation. This may be accomplished by filtering solution *B* into the cylinder, arranging the funnel so that the solution runs slowly down the cylinder wall, thus not disturbing the interface.

The space between the 2 cylinders is filled with the dilute acid copper sulfate.

The density gradient is established by partially mixing the 2 layers with the spiral stirrer, G, in the following manner: Churning strokes of uniform velocity are made with the spiral between positions 3 or 4 cm. above and below the middle graduation until the *schlieren* effects (disturbances in transmitted light due to differences of refraction) are of nearly the same magnitude throughout the interval. The strokes are then lengthened by steps of 6 or 8 cm. until they reach the full length of the column. At each step, mixing is continued until the *schlieren* effects are of the same magnitude throughout the interval. It will perhaps be surprising to find how much stirring this requires. The usual mistake is to stir too little rather than too much. After 5 or 10 minutes, droplets of each of the standard solutions are put into the cylinder and the positions at which the droplets stop are noted. The total interval between the lightest and the heaviest standard should be one-half to one-fourth of the graduated length of the cylinder. The standard droplets should be nearly equally spaced (± 20%). If the droplets are too close together, more full length strokes should be made. If the gradient does not approximate linearity, it may be brought into linearity by stirring more when the standards are too close together, although this will seldom be necessary. If the droplets are too widely spaced, there has been too much stirring. In this event the gradient should be reestablished as follows: The bromobenzene and kerosene are siphoned or poured out, completely mixed, and divided into 2 equal parts. Each part will now have a specific gravity of about 1.03. To 1 part are added 95 ml. of bromobenzene/l. to bring it to 1.07 specific gravity. To the other portion are added 210 ml. of kerosene/l. to bring it to a specific gravity of 0.99. With these as the starting solutions, the cylinder is filled as before and the gradient is reestablished as described above.

After the cylinder has stood for half an hour, the gradient is ready for use. It should be set on a level table out of direct sunlight and away from other sources of heat. A light background will facilitate reading. The gradient will improve with time, becoming more nearly linear. Eventually, the intervals between standards will become greater and finally, after 6 months to a year, depending on use and protection from evaporation, the heaviest standard will fall through to the bottom. When this occurs, the gradient should be reestablished as described above. In warm weather, the standards will all lie lower than in cold weather, since the temperature coefficient of water is much less than that of the organic solvents. If the gradient tube is not kept tightly stoppered when not in use, the more volatile bromobenzene will evaporate somewhat.

D. USE OF GRADIENT TUBE

The constriction pipette (Fig. 3, C) is rinsed 2 or 3 times and filled to the upper constriction with the heaviest standard, a rubber tube being used as with a blood-counting pipette. The pipette tip is gently wiped on a slightly moistened piece of filter paper or towel and introduced into the gradient tube on the extreme left side. With the tip just below the liquid surface and about 5 mm. from the wall, the solution is blown down to the middle constriction where surface tension stops it. By raising the tip of the pipette through the surface, the droplet pulls away and falls. The tip is put back into the liquid and the second droplet is delivered a few mm. behind the first. Care is taken not to blow air after the second droplet, as this would break the droplet into a fine spray.

The rest of the standards are put directly on top of the first in the order of decreasing specific gravity. The pipette is rinsed several times with each new standard before use. For nearly normal sera the 5.5, 7.0 and 8.5% protein standards will suffice. The positions of the standard droplets are read 4 minutes or more after the last standard has been introduced, by which time all will have reached equilibrium. It is more convenient and accurate to read the position of the lower edge of each droplet than to try to estimate the height of its center.

Serum is delivered in the same manner as the standards. Separate pipettes should be used for the standards and for serum, since serum leaves a film on the pipette which makes it difficult to pipette salt solutions. If there is insufficient serum available to rinse the pipette, it may be rinsed with water and dried with acetone before use. There is ordinarily little or no error if different sera are pipetted one after another without rinsing. If a pipette that has been used for serum remains idle for more than a moment, it should be rinsed with water, since otherwise the fine tip may dry shut. A dried tip can usually be reopened with concentrated HNO_3.

The serum droplets are delivered into the gradient tube a few mm. to the right of the standards. A duplicate droplet is unnecessary if there is a shortage of serum, but its presence increases confidence in the result and adds scarcely at all to the time consumed. Each serum is introduced in a separate "lane" a few mm. to the right of the previous one. Vertical marks at the top of the cylinder aid in placing the droplets in separate lanes. As many as 20 or 30 serum samples may be inserted if care is taken to place them close to each other. If desired, a second row of droplets may be inserted 2 or 3 cm. behind the first. Each pair of droplets is read 4 ± 0.5 minutes after delivery. In the interval, the next 3 or 4 samples may be put in. With experience, no difficulty is encountered in keeping track of the time and order of the droplets. The positions of the standards are plotted on graph paper against their equivalent serum protein concentration, and from the resultant curve the unknown sera are evaluated. It is unnecessary to consider the temperature of the gradient tubes at all, since both serum and standards have essentially the same temperature coefficient.

E. REMOVAL OF DROPLETS

After the column becomes full of droplets a small amount of sea sand is sprinkled into the top of the gradient tube. The fine particles adhere to the droplets, carrying them to the bottom, and the gradient may be used again immediately. Although there is usually no change, a new set of standards should be read before making a new series of measurements.

F. TURBIDITY

If the gradient tube should ever become turbid (due to moisture), it may be clarified by grinding a little $CaCl_2$ with a few ml. of the top gradient mixture and quickly pouring the resultant suspension into the column. It will be necessary to wait some hours after this before using the gradient again, to give time for the finer particles of $CaCl_2$ to settle out.

G. REPRODUCIBILITY AND COMPARISON WITH KJELDAHL METHOD

Replicate measurements on the same specimen should deviate by not more than 0.02 g.-% protein. Specimens collected simultaneously from a vein, finger puncture and ear puncture agree within 0.1 g.-% (1). It is interesting that squeezing the finger while collecting the specimen does not influence the results. This suggests that the well-known influence of squeezing on hematocrit values is due to dilution with serum rather than to dilution with tissue extracellular fluid of lower protein concentration.

The serum protein values of 240 patients ill of a variety of diseases, excluding obvious liver damage, have been measured by both the gradient tube and Kjeldahl method by Lowry and Hunter (1). The serum protein concentrations varied from 3.5 to 13.5 g.-%. A standard deviation of only 0.24 g.-% was found between the 2 methods, and in only 5% of the cases did the values deviate by more than 0.4 g.-%. Deviations between the methods on specimens from normal population groups would, of course, be considerably less than the above figures.

The gradient tube shares, in common with all other serum protein methods based on measurement of specific gravity, the disadvantage that serum constituents other than protein influence serum density. It should be noted, however, that, in order to influence the apparent serum protein concentration by as much as 0.1 g.-%, it would be necessary to double the normal concentration of serum lipides, the blood glucose, or the nonprotein nitrogen. It is evident that it is rare for any of these factors to affect seriously the results.

H. STABILITY

Serum from whole blood stored in the collecting tubes at 4° C. has been found little changed after 48 hours. At 30° C. a definite increase in serum density occurred in 25 hours, presumably due to the consequences of the swelling of red cells with attendant concentration of the serum, resulting from glycolysis. Separated serum stored in tightly stoppered tubes may be left satisfactorily for a week or so at 4° C.

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Microbiological Methods in Vitamin Research

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I. Introduction

Within the last few years, microbiological methods for the determination of vitamins have come to be widely used. At present, they occupy a very important position among the methods available for this purpose, and some knowledge of the inherent advantages and limitations of the methods, and of the fundamental principles upon which they rest, is important for everyone who deals with the biochemistry of the vitamins.

Just as the use of experimental animals for the determination of vitamins grew out of a study of their nutritional requirements, so the use of microorganisms for this purpose arose from more fundamental studies dealing with their metabolic requirements. As a result of such studies, a striking similarity between the nutritive requirements of animals and microorganisms became apparent. Indeed, the study of the nutrition of microorganisms has proved to be an excellent approach to the study of animal nutrition (1, 2). Inositol (3), pantothenic acid (4, 5, 6, 7), biotin (8), nicotinic acid (9), *p*-amino-benzoic acid (10, 11), pyridoxal and pyridoxamine (12, 13, 14), are examples of substances whose essential nature for microorganisms was discovered before their role in animal nutrition was recognized. The important role played by the lactic acid bacteria in the discovery, isolation and characterization of various naturally-occurring forms of folic acid (14a, 15, 16, 17, 18) is well known. A further example of the usefulness of these microorganisms in this field is provided by their use as test organisms in the recent isolation of vitamin B_{12} (19, 20). In many cases, the growth-promoting action of these substances for microorganisms provides the only rapid and reasonably specific methods presently available for their determination. Although this chapter deals almost exclusively with quantitative aspects of this latter application, the important contribution which studies in microbiological nutrition have made to the discovery and purification of the vitamins should not be forgotten. The nutritional requirements of only a comparatively few species of microorganisms have been thoroughly investigated (reviews: 21, 22) and further study in this field should result in discovery of additional trace substances necessary in metabolism. These may or may not prove to be dietary requirements of higher animals as well.

While microorganisms have been used since comparatively early times to detect qualitatively or semiquantitatively substances which promote their growth, their routine use for quantitative estimation of such substances is of more recent origin. Quantitative methods for the estimation of available phosphates and potassium in soil with *Azotobacter* (23) or molds (24, 25) were developed between 1930 and 1934. The use of yeast for the determination of "water-soluble vitamine" had been suggested much earlier (26), but knowledge of the detailed nutritional requirements of yeast or other microorganisms did not permit such use until a much later date. A rapid expansion in our knowledge of nutrition of microorganisms began about 1936. As the organic factors required for growth of a given organism were identified, it became possible to use the growth response of that organism to additions of the growth factor as a test for the presence of the latter in natural materials. Further experience with such a test often permits, through successive modifications, eventual development of a satisfactory quantitative method. One of the first such methods to be widely used was that developed by Snell and Strong (27) for the determination of riboflavin. It arose by modification of a previous simpler medium (6) which proved unsuitable for the quantitative determination of riboflavin in natural products, although it did permit excellent growth responses to riboflavin and riboflavin-containing materials. That assay methods develop gradually by trial, modification and retrial, sometimes several times repeated, although obvious on consideration, is often overlooked in practice. As another example, the early yeast growth method for pantothenic acid (28) may be cited. This method, by means of which pantothenic acid was originally discovered, and which served admirably as a guide for purification and near isolation of this substance, is completely unsatisfactory for its routine determination in natural materials (29). Subsequent studies have permitted development of a modified medium which permits much more accurate determinations of pantothenic acid with yeast (30).

In the sequel, a large number of methods for the determination of the various vitamins will be described. Some of these have been adequately tested, and are known to give accurate results when applied to samples of great diversity. Others have not been so widely tested, and may be properly classed as research methods. Results of their routine application to natural products should be carefully checked for accuracy.

Microorganisms are known which require external supplies of one or more of each of the water-soluble vitamins with the exception of ascorbic acid. If approximately optimal conditions for growth of such an organism are selected, and a medium is supplied which contains each of the substances required by the test organism except that to be determined, the essential conditions for a microbiological assay are met. Under these conditions, the amount of growth of the test organism is, within limits, a function of the amount of the missing vitamin supplied in the medium. From the response of the organism after a definite time interval to increasing concentrations of the pure vitamin, a "standard curve" may be constructed. The sample is also assayed at several concentrations calculated to yield responses which fall upon the standard curve. The most common method for calculating the amount of vitamin in the sample is to interpolate these responses onto the standard curve. An average of the equivalent values in terms of concentrations of the pure vitamin is taken as the vitamin content of the sample.

For such assays, accurate methods for determining response of the organism to additions of the vitamin must be available. Detailed procedures vary, and will be described with individual methods of assay; all procedures fall, however, into two general classes: growth of the organism is either determined directly (*e.g.*, measurements of turbidity, weight of dry cells, amount of cellular nitrogen, direct cell counts, *etc.*), or the amount of some metabolic product of the organisms (acid, CO_2, *etc.*) is measured. Whatever method is used, comparison of the response to various concentrations of the test substance may be carried out before or after growth or fermentative activity has stopped. In the former case, one measures comparative *rates* of response; in the latter case, the *total* response permitted by a given concentration of the essential substance is measured. With any given organism, assays based on total response are likely to be more reliable than those based on rate of response, since at present it is difficult to devise a basal medium so complete or well-balanced that substances present in crude samples will neither stimulate nor suppress the rate of response to the essential substance being measured. Since such substances are not required by the test organism, they do not affect greatly the total response to limited amounts of an essential substance. For these reasons, it is usually more difficult to devise a short term assay than to devise a longer term assay of equal accuracy. Most of the methods to be described later measure total response rather than rates of response.

With every method of assay, it is important to know whether the method is giving reliable results. Various criteria in use for this purpose have been rather fully discussed elsewhere (30a, 31). Of greatest importance is that values obtained by the proposed microbiological method check those obtained by other reliable methods. It is usually true, however, that the reliability of other available methods is established no better than that of most microbiological methods. In such cases, one must rely upon indirect evidence of an internal nature. Such evidence includes ability to secure quantitative recoveries of the added vitamin, and to obtain consistent values upon repeated assay. Perhaps the best evidence for reliability is obtention of values for the vitamin content of the sample which check when calculated from each of the several concentrations at which the sample is assayed, and which show no consistent trend (or "drift") either up or down with increasing assay level (see also page 350). Such behavior demonstrates that the assay organism responds in exactly the same manner toward the pure vitamin used in establishing the standard curve and the sample, *i.e.*, that extraneous materials added with the sample are neither stimulating nor suppressing the response to the vitamin. Additional evidence for reliability can be obtained when several different organisms which require the vitamin but which differ in other respects are available. If assay values obtained with two or more such organisms agree, the probability that the value is correct is greatly increased, the more so the more distant the relationship between the organisms used.

It is well known that some microorganisms which appear to require a given vitamin for growth may be able to synthesize it from precursors which are unavailable to higher animals or to other microorganisms. Since one major purpose of most vitamin determinations is to assess the value of a sample as a dietary source of the vitamin for animals, it is important that the specificity of the response of the test organism to such compounds be known. In many cases, too, the vitamins occur naturally in combined forms which animals are able to break down and utilize, but which are unavailable to microorganisms. A most pressing need is for adequate extraction methods which will render such combined forms available for use of the test organism. Much of the dissatisfaction expressed with certain microbiological methods arises not from failure of the method *per se,* but from lack of suitable procedures for liberating the vitamin in a utilizable form. Although this very difficulty is most useful, in that it permits tests to be devised by means of which hitherto unknown combined forms of the vitamins may be recognized and eventually isolated, it is not a satisfactory situation from the standpoint of the analyst.

The problem of adequate extraction is shared, of course, by both microbiological and chemical methods. With animal assays, although it is not certain in all cases, extraction and utilization of the limiting vitamin is presumably complete. In this respect, animal bioassays have an advantage over microbiological or chemical methods, especially since the chief application of the findings is to animal nutrition. In most other respects, microbiological assay is preferable to animal assay. Results can be obtained in much less time, with far smaller amounts of sample, and in most instances with greater precision. The saving in time, expense, and labor is tremendous. These advantages are shared to some extent by chemical methods. At present, however, the number of vitamins for which adequate chemical methods are available is limited.

The statement has often been made that microbiological methods cannot be expected to vary less than ± 10 or 15% from an average figure because of the "biological variability" of the living organisms employed.

While this is probably true of assays employing higher animals, where only a limited number of animals can be used because of operational or budgetary difficulties, there is no reason to accept the conclusion with microbiological assay methods, where the response observed in each assay tube represents a true statistical average of the activities of millions of organisms. Although microbiological assays for vitamins presently in use are usually not more accurate than this, the variability should be ascribed to inadequacies in the media used or to inadequate control of other factors connected with the assay, rather than to an innate variability of the assay organisms used. That this is actually the case is shown by the careful work of Lowry and Bessey (32), who showed that pure riboflavin could be determined by a microbiological procedure with a probable error of only 0.7-1.2%.

II. Methods Utilizing Lactic Acid Bacteria

1. Nutritive Requirements of the Lactic Acid Bacteria Used in Assay Work

The nutritional requirements of various lactic acid bacteria used in assay work are well known and have been summarized elsewhere (22, 30a, 33, 34). Cultures are known which require one or more of the following vitamins (Table I): thiamine, riboflavin, nicotinic acid, pantothenic acid, biotin, vitamin B_6, folic acid, and *p*-aminobenzoic acid; and assay methods for each of these substances which employ these organisms have been devised. In addition, lactic acid bacteria require an assortment of amino acids for growth (30a, 31) and often additional substances, such as purine and pyrimidine bases. This complex nutrition is advantageous in that it permits use of very similar techniques and often a single organism for the determination of a large variety of biologically important substances. It may also be disadvantageous in some cases, since basal media for use with such organisms must often be more complex than those suitable for use with less fastidious organisms.

In addition to suitable sources of the amino acids and vitamins, media for use with the lactic acid bacteria must contain a fermentable carbohydrate, mineral salts, and a buffer. The latter is necessary because, in poorly buffered media, these organisms rapidly produce sufficient acid to lower the *p*H to levels which inhibit their further growth.

2. The Preparation of Stock Cultures and Inocula

Stab cultures of the assay organism have been most often carried in a solid medium which contains an extract of yeast, glucose, and agar, with

TABLE I

Vitamin Requirements of Various Species of Lactic Acid Bacteria[a]

Organism and Strain No.	American Type Culture Collection No.	Reference	Biotin	Folic acid	Nicotinic acid	Pantothenic acid	Riboflavin	Thiamine	Vitamin B_6
Lactobacillus arabinosus 17-5	8014	(33)	+	—	+	+	—	—	—
Lactobacillus casei Bc-1	7469	(33)	+	+	+	+	+	—	+
Lactobacillus delbrueckii 3	....	(33)	+	+	+	+	+	—	+
Lactobacillus fermenti 36	9338	(33, 35)	+		+	+	—	+	
Lactobacillus pentosus 124-2	....	[b]	+	—	+	+	—	—	—
Leuconostoc mesenteroides P-60	8042	(33)	+	—	+	+	—	—	—
Leuconostoc mesenteroides	9135	(36, 37)	+	—	+	+	—	+	+
Streptococcus faecalis R[c]	8043	(33)	+	+	+	+	—	—	+
Streptococcus lactis	....	(38)	+	—	+	+	—[d]	+[d]	—

[a] A "+" sign indicates that the vitamin is required for growth of the organism, a "—" sign that the substance is not required for growth. Frequently, a nonessential substance stimulates rate of growth, although the final amount of growth obtained is not affected.

Only two organisms of this group, *Lactobacillus arabinosus* (39, 40) and *Leuconostoc mesenteroides* P-60 (41), have been reported to require *p*-aminobenzoic acid; this requirement appears to be very readily lost, so that most of the cultures of these organisms in use do not respond to additions of this substance.

[b] Unpublished data.

[c] Niven and Sherman (42) examined a large number of enterococci and found only 7 of 19 which required folic acid. On the other hand, 17 of the 19 required riboflavin. In both respects, the culture listed in the table is not typical of the group. All cultures required biotin, nicotinic acid, pantothenic acid, and vitamin B_6. The result emphasizes the variation which may exist between different strains of a single species.

[d] Of 21 strains tested, one-third required riboflavin; 18 required thiamine. Examinaton of a number of strains of the other species listed would probably show similar variation to occur in these cases as well.

or without small amounts of added buffer. The composition of some representative media of this type is given in Table II. Different investigators have varied the proportions of the various ingredients in this medium slightly, but whether such variation significantly affects the behavior of the organism in subsequent assays has never been determined; certainly, the exact composition of the medium is not critical. Experience of various workers indicates, however, that prolonged exposure of these organisms to high acidity weakens them. Development of such acidity along the line of the stab can be avoided by using a low concentration of sugar in the medium and by using an adequately buffered medium. Medium c (Table II) which contains sodium acetate, has much to recommend it on both counts. In his extensive early work with a large number of different species of these organisms, Orla-Jensen (43) recommended that only 0.25% glucose be used in media for carrying stock cultures. A recent paper (44) recommends still less, 0.1%. Development of high acidity can also be prevented by incubating the stock cultures only long enough to get distinctly visible growth along the line of the stab (usually 16 to 24 or 36 hr.), then transferring to the refrigerator, which is held at temperatures between 5° and 18° C. where metabolic activity is at a minimum. Whatever concentration of acid exists along the stab at this time rapidly decreases as the acid diffuses away from the stab into the unused medium.

TABLE II

Composition of Media for Carrying Stock Cultures of Lactic Acid Bacteria

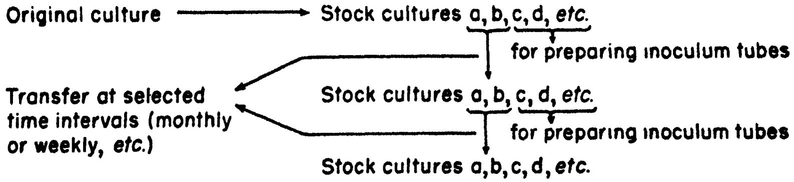
Reference	Yeast extract (Difco) *Per cent*	Glucose *Per cent*	Agar *Per cent*	Sodium acetate *Per cent*
a 27, 45, 46	[a]	1.0	1.5	—
b 47, 48, 49	1.0	1.0	1.5	—
c 50	1.0	0.5	1.5	0.5
d 35, 51	1.0	1.0	2.0	—

[a] "Yeast water" was used here in place of dehydrated yeast extract. This is prepared by heating fresh, starchfree baker's yeast with 10 times its weight of distilled water at 100° C. for 2 hr., then at 15 lb. pressure (121.5°) for 40 min. The mixture is allowed to settle, then filtered (46). Appropriate amounts of glucose and agar are dissolved in this extract to give the composition indicated. A simple procedure is to distribute suitable volumes of the yeast and water mixture to bottles or flasks in which the heating can be carried out. These are plugged with cotton so that the mixture is sterile after heating, and will keep indefinitely in the dark. As required, a flask is opened, filtered, and used for preparation of the medium.

How frequently stock cultures should be transferred for optimum results has never been adequately investigated. In the early procedures (*e.g.*, 27, 35, 45, 48, 43) monthly transfer was used; the author has maintained vigorous cultures of most of the lactic acid bacteria presently used for assay for almost 10 yr. by this procedure. Orla-Jensen (43) found that many cultures of lactic acid bacteria could be kept alive for several years—without transference—in similar media, and has kept many cultures longer than 30 yr. by employing monthly transfers (52). More frequent transfer (at biweekly (51) or weekly (32, 46, 53, 54) intervals) has been favored by many later investigators. Again, the exact procedure followed certainly is not critical; however, the common bacteriological practice of carrying a culture through several successive transplants when particularly active organisms are desired indicates that, in at least some cases, frequent transfer may be the preferable procedure (*cf.* 55).

In a recent study (54), it was found that the magnitude of the response of two commonly used assay organisms, *L. arabinosus* and *L. casei,* to limiting amounts of nicotinic acid or of riboflavin decreased after long periods of time if stock cultures were transferred monthly on media of the type shown in Table II. Such cultures could be "rejuvenated" by a series of transplants in an enriched medium containing 1 g. of tryptone (Difco), 1 g. glucose, 0.2 g. K_2HPO_4, 0.3 g. $CaCO_3$, 1.5 g. of agar, 0.5 cc. of Salts A and B (27) (for composition see footnote to Table IV), and the water extract from 2.3 g. of fresh liver per 100 cc. of medium. These authors believe yeast-glucose agar is deficient in certain nutrients necessary for maintenance of the most active cultures of these test organisms, and recommend transfer of stock cultures at weekly intervals in the modified medium.

Whatever medium is adopted, approximately 10 cc. portions of the hot solution are added to test tubes. These are plugged with nonabsorbent cotton, sterilized by autoclaving at 15 lbs. steam pressure for 20 min., and allowed to solidify in an upright position. Stab cultures of the desired organism are prepared in as many such tubes as are required for use during the period before the next transfer is made. These are incubated for 16-36 hr. at the appropriate temperature (see section 3 below), then stored in the refrigerator until needed. One or more such stab cultures is designated as the stock culture (a, b), and is not used except to transfer at the selected interval. The other stabs (c, d, *etc.*) are used for the preparation of inocula from day to day (see diagram).


Original culture
Stock cultures a, b, c, d, *etc.*
for preparing inoculum tubes
Transfer at selected time intervals (monthly or weekly, *etc.*)
Stock cultures a, b, c, d, *etc.*
for preparing inoculum tubes
Stock cultures a, b, c, d, *etc.*

Usually, a medium for the inoculum culture is prepared from the basal medium for the assay being carried out by supplementing it with sufficient of the missing vitamin to permit adequate growth of the assay organism, although, in some cases, an entirely different, enriched medium may be used. To prepare inoculum, a transfer from a stab culture (c, d, *etc.*) is made into 10 cc. of this inoculum medium. After 16-24 hr. incubation at the temperature used for assay, the inoculum is ready for use. Subsequent treatment depends upon the individual procedure followed, and will be described with each method.

3. THE INFLUENCE OF TEMPERATURE, *p*H, OXYGEN, AND CARBON DIOXIDE ON GROWTH OF LACTIC ACID BACTERIA

As a general rule, any organism used for assay should be cultured at or near its optimum temperature. This varies considerably for different organisms of the lactic group from around 20° C. for many strains of *Streptococcus cremoris* to as high as 50-60° C. for *Lactobacillus thermophilus* (56). The cultures thus far used for assay work are listed (56) as having temperature optima over a much narrower range, 30-37° C. It should be realized, however, that different strains classed together as representatives of a single species do not necessarily have the same temperature optimum. Thus *L. casei* is stated (56) to have a temperature optimum of 30° C. The strain of this organism used in the determination of riboflavin (27) is atypical in a number of respects (57) and has usually been grown at 37° C., although the true temperature optimum for its growth may be even higher, at 41-42° C. (58). This organism grew well in a complete medium at temperatures of 24, 30, 40 and 50° C., and produced approximately equal amounts of acid at the latter three temperatures during a 3-day incubation period (59). A comparison of the assay values for valine obtained with this organism at 32.5° C. and at 37° C. showed no difference at the two temperatures (60). Similarly, *Lactobacillus arabinosus*, listed as having its temperature optimum at 30° C. (56, 61) has been widely used for assay of nicotinic acid at either 30° C. or 37° C. (46, 48, 50) with no detectable differences in the accuracy of the results obtained at the two temperatures. The exact temperature selected for incubation is thus apparently not of crucial importance, as long as it lies reasonably near to the optimum temperature, in a range which permits good growth of the test organism. It is of primary importance, however, that the temperature selected for incubation be maintained constant throughout the area occupied by the assay tubes. Price and Graves (58) showed that temperatures in a small air incubator varied 3-4° from 37° C. at different locations in the incubator, and that, as a result, the response of *L. casei* to a constant level of riboflavin ($0.075\gamma/10$ cc.) following the customary 3-day incubation period varied from 3.4 to 4.9 cc. of $N/10$ acid. Rigid temperature control is thus essential for accurate microbiological assays; it becomes increasingly more important the shorter the incubation period used with a given medium and organism. In air incubators constant temperature is most easily obtained by forced circulation of the air; a well-stirred water bath, held within ± 0.1° of the selected incubation temperature is even more effective and is recommended for all careful work.[1] Temperatures which have been recommended for use with the various assay organisms are as follows:

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[1] Recent work has shown that, in short term assays where comparative rates of

Lactobacillus arabinosus: 30-37° C. with little preference expressed.
Lactobacillus casei: 32.5-42° C., most commonly at 37° C., but higher temperatures not adequately tested.
Lactobacillus fermenti: 37° C.; effect of variation not adequately studied.
Leuconostoc mesenteroides: 21-35° C.; effect of variation not adequately studied.
Streptococcus faecalis: 30-37° C.; effect of variation not adequately studied.

In many instances, cultures of these organisms are known to grow at temperatures both above and below those used for assay, and it is quite possible that superior assays could be obtained in some cases at temperatures outside the ranges thus far recommended.

The optimum *p*H for growth of most lactic acid bacteria is stated (52, 63) to lie between 5.5 and 6.5. As some acid production occurs during autoclaving, the initial *p*H of the medium before sterilization should be somewhat higher than this, 6.8-7.0 (63). This is in accord with common practice. It has been indicated (64) that the quantitative response obtained to various levels of a vitamin differs with the *p*H of the medium. It is, therefore, of great importance that all of the samples for assay be adjusted to the same initial *p*H as the medium. Similarly, the buffer content of the sample must not be so high that it materially changes the buffer capacity (or the buffer range) of the medium.

As the organisms grow, the lactic acid produced lowers the *p*H of the medium, eventually to levels which inhibit further growth of the bacteria. The *p*H which thus limits growth varies with different organisms from just over 4 to as low as 3 (65). In general, the homofermentative rod forms (*e.g., L. casei, L. arabinosus, L. delbruckii, etc.*) tolerate the highest acid concentrations; the coccus forms (*e.g., S. lactis, S. faecalis, Leuco. mesenteroides, etc.*) are considerably more sensitive to acid (43, 52). In all cases, extra buffers are added to assay media to permit heavy growth and high acid production to occur before the *p*H is lowered to inhibitory levels. From the above relationship to *p*H it is obvious

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growth are measured, and where adequate temperature control requires use of a water bath, care should be taken that circulation of the bath water does not impart variable vibration to the different tubes of an assay, since under these conditions the variable agitation of the medium of different tubes may result in differences in growth which are just as large as those produced by minor temperature differences in different parts of the bath (62).

that the buffer requirement for high acid production by *S. faecalis*, for example, will be greater than that of *L. arabinosus*, so that the type and quantity of buffer used will depend in part upon the test organism. Orla-Jensen (52) has shown that the final *p*H produced in media of low buffer capacity may actually be lower than that produced in adequately buffered media, although much more acid is produced in the latter media. The failure to achieve the same low *p*H values in both instances is attributed to the larger amounts of lactate which accumulate in well-buffered media, and which is itself somewhat toxic to these organisms.

Acetate is the most commonly used buffer for these organisms. For many species, small amounts of this substance are necessary for rapid growth (66), which explains in part, why other buffers are generally less suitable than acetate (6, 27, 32, 66). If the small amounts of acetate necessary for adequate growth were furnished, it is quite possible that other buffers would, in some cases, prove superior to acetate. With *S. faecalis*, for example, which does not require acetate for rapid growth, citrate or phosphate is a more effective buffer than is acetate (67).

The lactic acid bacteria show a relationship to oxygen which especially suits them for assay work. The rod forms (genus, *Lactobacillus*) are almost uniformly microaerophilic, while the coccus forms (genus, *Streptococcus, Leuconostoc*) are facultative aerobes. In practice, this means that all of the organisms will grow uniformly throughout a deep tube of medium, and that aeration, by shaking or otherwise, is inadvisable with the rod forms, and unnecessary for the cocci. As the assays are usually carried out, no special precautions are required to maintain anaerobic conditions of growth, since a favorable oxidation-reduction potential for growth results from autoclaving the carbohydrate-containing media. Under special conditions, however, or with certain organisms, oxygen may be deleterious. Thus, in carrying out riboflavin determinations with *L. casei* on a micro scale (0.2 cc. of culture medium in place of 10 cc.) it was found helpful to "refresh" old media by addition of cysteine, and to incubate the assay in an atmosphere of CO_2 instead of air (32). Similarly, in assays for thiamine with *S. salivarius*, addition of thioglycollate to the medium is necessary to obtain uniform results (68). On the other hand, addition of ascorbic acid to the medium is reported (69) to eliminate the requirement of *Lactobacillus lactis* for vitamin B_{12}. In special instances, therefore, it may be of importance to control the oxidation-reduction potential of the medium rather carefully. Generally this is unnecessary.

Although, under ordinary conditions of assay, the lactic acid bacteria do not require increased CO_2 tensions for continued growth or for initiation of growth, it has been shown to affect their growth under special conditions. Thus, acid production by *L. casei* was stimulated by incubation in an atmosphere of CO_2 when the organism was cultured on a micro scale (32). Lyman and coworkers (70) found that, in an otherwise complete medium and in an atmosphere containing 6% of CO_2, both *L. arabinosus* and *S. faecalis* could dispense with certain amino acids which were essential for their growth in the absence of CO_2. Except in special cases, however, little attention need be paid to CO_2 tension.

4. Assay Methods for Individual Vitamins

All of the assay methods which employ lactic acid bacteria are similar, regardless of the organism being used or the vitamin being determined. In every case, the appropriate basal medium, free of the vitamin to be determined, is prepared at twice its final concentration. Standard solutions of the pure vitamin and of the samples are added to a series of uniform test tubes in amounts suitable to produce gradations in growth between no growth, and maximum growth. Contents of all tubes are diluted with water to the same volume, and an equal volume of the medium is added. The tubes are covered with glass or aluminum caps, or plugged with cotton, sterilized by autoclaving, cooled, and inoculated with a suspension of the test organism obtained from an actively growing culture. The assay tubes are incubated at a constant temperature near the optimum for the test organism, preferably until growth or acid production has reached the maximum permitted by the limiting vitamin present. The response to standard and sample is then determined, either by titration of the acid produced in each tube, or by measurement of culture turbidities in a photoelectric comparator. The response to each dose of the pure vitamin is then plotted against the concentration to produce a standard curve. Ordinarily, the amount of vitamin present at the various testing levels of the samples is determined by interpolation of the response to them onto the standard curve. From simple dilution factors, the amount present in the original sample is then calculated. The results secured at several levels should agree closely, and are averaged to obtain the final result.

Under certain favorable conditions, a modified method of calculation can be employed which permits an exact determination of the standard error of the result. This will be described in greater detail below.

Such procedures, as they apply to assay of riboflavin and of nicotinic acid, will be given below, both in detail and in summarized form. For the other vitamins, summarized outlines of the technique will be given, with the significant variations indicated. Where questions of the mechanics of assay arise, reference should be made to the detailed procedures for riboflavin and nicotinic acid.

In comparing the merits of two assay media, the same extraction procedure should be used in both cases, for, if inadequate extraction procedures are employed in preparation of the sample, no assay medium, however good, can yield reliable values. For this reason, it is believed that extraction procedures should be considered as a separate phase of the problem of microbiological assay and this plan will be followed in describing the individual methods below.

A. RIBOFLAVIN

(1) Extraction from Natural Materials

All operations with riboflavin or riboflavin-containing materials should be carried out in subdued light or in low-actinic glassware, since this vitamin is destroyed by light (71), especially at elevated temperatures (72). Even subdued daylight may be destructive, but artificial light of an intensity of 6 candles/sq. ft. or less is permissible (73). Riboflavin is also rapidly decomposed by alkali (72); which should be carefully avoided in the preparation of all sample and standard solutions. Even clean glassware may have sufficient alkali on its surface to inactivate small quantities of riboflavin, and where extremely dilute solutions of riboflavin are used in receptacles having a high surface to volume ratio (as occurs in an ultramicro assay for riboflavin (32)) such inactivation may be a significant source of error unless eliminated by use of acid-washed glassware (32). In contrast, riboflavin is comparatively stable toward acid treatment, and, to avoid the neutral or alkaline conditions which promote its destruction, standard solutions of this vitamin are generally prepared in 0.02 *N* acetic acid.

Pure riboflavin is stable toward most oxidizing agents, and these frequently have been used to destroy interfering pigments in natural extracts, preliminary to chemical estimation of the vitamin. However, the stability of riboflavin in natural extracts to action of these reagents should not be assumed. For example, riboflavin is decomposed rapidly by dilute solutions of H_2O_2 in the presence of traces of ferrous iron, although it is stable to the former reagent in pure solution (74).

For extraction of riboflavin from natural materials preliminary to microbiological estimation, variations of three procedures have been recommended. These are: (a) autoclaving the finely divided material with large volumes of water (27) or (b) *N*/10 acid (27, 46, 75, 76), and (c) digestion with enzymes followed by boiling or autoclaving with water (77, 78, 79, 80). Although the "bound" riboflavin of natural materials is mostly liberated simply by heating (71), it is found in practice that more consistent and slightly higher values are obtained if the sample is extracted by heating with $N/10$ HCl, and this procedure has been recommended most widely. The volume of extractant which can be used is limited only by the toxicity of NaCl (formed when the sample is neutralized) for the test organism. Disturbing effects of NaCl begin at concentrations of 50-100 mg/10cc. of medium (27), so that, even for low potency samples (*e.g.*, containing 1 γ of riboflavin/g.), the ratio of extractant to sample can be maintained as high as 20 to 1. It is important that this ratio be maintained fairly high (say 10:1 as a minimum) if complete extraction of riboflavin is desired. Recent work suggests that high extraction ratios may be maintained even with low potency samples if KOH rather than NaOH is used for neutralization of the extract (81).

Enzymatic digestion of certain samples with papain and takadiastase was found by two independent groups (78, 79) to yield results as high as, or higher than, those obtained by acid-autoclaving. Enzymatic digestion, but not autoclaving with $N/10$ acid, gave results which checked the high values obtained fluorometrically, following extraction with an acid-acetone mixture (79). These results indicate that enzymatic digestion procedures should be given greater consideration for use than has been customary in the past.

In all cases, traces of fatty materials present in the extracts must be removed, since they interfere markedly with the assay (76, 82, 83, 84, 85, 86). This is most easily accomplished by filtering the extract at *p*H 4.5 (76, 83), although extracts from samples rich in fats may require in addition ether extraction at *p*H 4.5 (76). In addition to free fatty materials, certain protein-like materials may occasionally cause comparatively minor disturbances in the assay. These can be removed by first acidifying the fat-free filtrate by adding HCl dropwise and filtering out any precipitate which appears, then readjusting to *p*H 6.8-7.0 by the dropwise addition of NaOH, and again filtering if a precipitate appears (87).

Starch is also reported to interfere with the assay to some extent, but its removal is easily accomplished by either of the recommended extraction procedures described below.

a. Recommended Procedure for Extraction of Sample with Acid. The following procedure is similar to that recommended by Strong and Carpenter (76). A convenient known weight of the finely ground or homogenized sample is suspended in not less than 8-10 times its weight of $N/10$ HCl. If the sample is high in riboflavin, larger quantities of extractant may be used. The mixture is autoclaved for 15-20 min. at 15 lbs. pressure (120° C.), cooled, and the *p*H adjusted to 4.5 by the addition of a 2.5 N sodium acetate solution. The mixture is diluted with water until the estimated riboflavin content is from 0.1 to 0.2 γ/cc., then filtered by pouring through a fluted, quantitative filter paper, repeating if necessary until a clear filtrate is obtained. Properly diluted aliquots of this filtrate, adjusted to *p*H 6.8-7.0, are used for assay.

b. Recommended Procedure for Enzymatic Extraction of Riboflavin. The procedure is essentially that described by Cheldelin, Eppright, Snell and Guirard (78). One g. of the finely divided sample is suspended in 8-10 cc. of 0.15 *M* acetate buffer, *p*H 4.5. To this is added 1 cc. of takadiastase suspension (20 mg./cc., dispersed in cold water) and 1 cc. of papain suspension (20 mg./cc.; the papain is first dispersed in an equal weight of glycerol, then diluted to 20 mg./cc. with water.) Both enzyme suspensions should be freshly prepared. To this enzyme-tissue suspension is added 0.5 cc. of toluene; the sample is shaken well, corked, and incubated for 24 hr. at 37° C. It is then diluted with water to an estimated riboflavin content of 0.1-0.2 γ/cc., readjusted (if necessary) to *p*H 4.5, autoclaved at 15 lbs. pressure for 15-20 min., cooled, and filtered as in (a) until a clear filtrate is obtained. With each lot of enzyme used, a "blank" determination, without sample, should be run to obtain the riboflavin content of the enzymes used. The result on the sample is then corrected for the riboflavin content of the enzymes. For most materials, this correction is usually negligible; for substances extremely low in riboflavin it may become appreciable, and for accuracy must not exceed 50% of the total value found.

(2) Assay Media for Riboflavin and Their Development

Orla-Jensen *et al.* (88), using alkali- or charcoal-treated milk as a basal medium, first showed that riboflavin was required by certain lactic acid bacteria. This finding was confirmed by Snell *et al.* (6), who later investigated the specificity of response of a number of different organisms of this group to riboflavin and certain related substances (89). The specific nature of this response suggested its use for the determination of riboflavin in natural materials. A strain of *Lactobacillus casei* was chosen as a suitable test organism, and a special riboflavin-free medium developed for use with it (27). The composition of the early medium (No. 1), and the assay medium developed from it (No. 2), is given in Table IIIa, together with that of a modified medium developed by Greene and Black (No. 3). The Snell-Strong medium (No. 2) has been widely used for assays. It is identical with that recommended by the Association of Official Agricultural Chemists (75). The U.S.P. XII medium (46) differs from it only in the concentration of glucose, which has been increased from 1.0 to 3.0%. The modified medium of Greene and Black (No. 3) permits considerably heavier growth than does that of

TABLE IIIa

Composition of Assay Media for Riboflavin

Constituents	Medium No. 1. Snell, Strong and Peterson, 1937 (6, 89)	2. Snell and Strong, 1939 (27)	3. Greene and Black, 1943 (90)
	g./100 cc. of double strength medium		
Peptone, alkali-treated	1.0	1.0	1.0
Yeast extract, lead acetate treated	—	0.2	—
Yeast extract, photolyzed	—	—	0.8
Sodium acetate	1.2	1.2	4.0-5.2[a]
Glucose	2.0	2.0	2.0
Cystine	0.02	0.02	0.02
Inorganic salts			
Salts A			
KH_2PO_4	.10	.10	.10
K_2HPO_4	.10	.10	.10
Salts B			
$MgSO_4.7H_2O$	.04	.04	.04
$MnSO_4.4H_2O$	.002	.002	.002
$FeSO_4.7H_2O$	.002	.002	.002
NaCl	.002	.002	.002
$(NH_4)_2SO_4$	—	—	4.0
	γ/100 cc. of double strength medium		
Pantothenic acid	20	—	—

[a] The original directions are not explicit as to the amount of sodium acetate used. By implication, 2% of sodium acetate is added to a medium containing the same quantity of sodium acetate as the Snell-Strong medium, which would make a final concentration of 2.6%, or 5.2 g./100 cc. of double-strength medium.

Snell and Strong, and in it the test organism is sensitive to somewhat smaller amounts of riboflavin. There is no apparent reason why it should not yield accurate results, but it has not been subjected to the test of widespread use. The same comment holds for the media described in Table IIIb, which, with one exception (No. 4), are stated by their originators to permit somewhat better growth or acid production than the original Snell-Strong medium. It should be emphasized that the principal objective of these methods is to secure accurate assays with a maximum of convenience, and not, necessarily, to secure the maximum possible growth or acid production. Data in support of claims for superiority of modified assay media have, in general, concerned themselves with the latter, rather than the former, point. While increased growth and acid production may result in increased accuracy, this is not necessarily true, as was shown in a recent comparison of Media 2 and 7,

TABLE IIIb

Composition of Assay Media for Riboflavin (continued)

Constituents	Medium No. 4. Landy and Dicken, 1942 (49)	5. Barton-Wright and Booth, 1943 (84)	6. Barton-Wright 1945 (91)	7. Roberts and Snell, 1946 (51)
	g./100 cc. of double strength medium			
Casein, acid hydrolyzed [a]	1.0	—	—	—
Casein, tryptic digest [a]	—	—	—	4.0
Peptone, NaOH-treated	—	1.0	1.0	—
Yeast extract, lead acetate-treated	—	0.2	0.4	—
Sodium acetate	1.2	1.2	1.2	4.0
Glucose	2.0	2.0	4.0	4.0
Xylose	—	0.1	0.2	—
L-Cystine	0.02	0.02	0.02	0.02
L-Tryptophan	0.02	—	0.02	—
Asparagine	0.05	0.05	—	—
	mg./100 cc. of double strength medium			
Uracil	1.0	—	2.0	2.0
Adenine	1.0	—	2.0	2.0
Guanine	1.0	—	2.0	2.0
Xanthine	1.0	—	2.0	—
	γ/100 cc. of double strength medium			
Pantothenic acid	40	50	20	100
Nicotinic acid	40	50	20	100
Biotin	1.0	—	—	0.8
Vitamin B_6 (pyridoxine)	80	—	20	200
Folic acid	0.3[b]	—	—	0.4
p-Aminobenzoic acid	—	—	80	20
Thiamine	20	—	—	100
	i.e., g./100 cc. of double strength medium			
Inorganic salts				
Salts A				
KH_2PO_4	.10	.10	.10	.50
K_2HPO_4	.10	.10	.10	.50
Salts B				
$MgSO_4.7H_2O$	.04	.04	.04	.16
$MnSO_4.4H_2O$	.002	.002	.002	.032
$FeSO_4.7H_2O$	.002	.002	.004 [c]	.008
NaCl	.002	.002	1.0	.008
$(NH_4)_2SO_4$	—	—	.6	—

[a] Purified ("vitamin-free") caseins are used in preparation of these hydroly-

Table III (51). These gave assays for riboflavin of equal accuracy, although Medium 7 was much superior in growth-promoting properties.

Media 4 and 7 represent attempts to devise a single medium which can be used for the microbiological estimation of any of the 6 vitamins, including riboflavin, required by *L. casei.* The vitamin to be determined is omitted from the medium, all others are added. Medium 4 has not been widely used since it is known to be deficient in naturally occurring substances which markedly affect growth of *L. casei* and which were not recognized at the time of its development. Some of these deficiencies are supplied in Medium 7, which has not been in use long enough to permit its proper evaluation.

Because of its widespread use, and because factors affecting its accuracy are most fully known, the Snell-Strong medium and procedure, essentially as modified in certain details by various collaborative studies, will be given below. As mentioned above, any of the modifications of this medium (No. 3, 5 and 6, Table III), in all probability, also will give satisfactory results; and to facilitate comparisons of them, the preparation of the modified yeast supplement used in medium 3 will also be described. The use of generalized media, similar to Nos. 4 and 7, will be discussed in a later section (pages 418-20).

(3) Reagents and Stock Solutions

a. Alkali-Treated Peptone Solution. To 40 g. of peptone[2] dissolved in 250 cc. of water is added 20 g. of NaOH also dissolved in 250 cc. of water. The solution is placed in the light in a crystallizing dish or plain glass bottle[3] and allowed to stand 24 hr.

Glacial acetic acid is then added to a pH of 6.6-6.8 (27-29 cc. are usually required), 7 g. of anhydrous sodium acetate are added, and the

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zates; the last traces of vitamins are removed from them by treatment with activated charcoal.

[b] The original paper recommended 2.0 γ of "50% pure folic acid." At that time, folic acid of "potency" 40,000 was assumed pure; it is now known that pure folic acid has a "potency" of 137,000 (92, 15, 93), and the amount actually added has been recalculated on this basis.

[c] $FeCl_3$ used in place of $FeSO_4.7H_2O$.

[2] Bacto-Peptone (Difco Laboratories, Detroit, Michigan) is suitable, and is most widely used. Different brands of commercial peptones may vary notably in source and composition, and since some growth essentials in the medium (other than amino acids, *e.g.*, folic acid (51) are supplied by the peptone, the suitability for use of new or unusual peptones should be checked against one of known utility. If a deficient peptone is used, its deficiences must be remedied by use of special supplements.

[3] The alkali-treatment alone usually suffices to free the peptone completely of riboflavin (*cf.* 6, 89), so that the conditions of exposure to light are not at all critical.

solution diluted with water to 800 cc. This stock solution contains 5% of treated peptone and 6% sodium acetate. It may be kept indefinitely under toluene in a refrigerator.

b. Lead Acetate-Treated Yeast Extract. This material may be prepared from fresh yeast (46), from autolyzed yeast (*e.g.*, Bacto Autolyzed Yeast, Difco Laboratories) (94), or from commercial, dehydrated yeast extracts (*e.g.*, Bacto Yeast Extract, Difco Laboratories) (27).

If the fresh yeast is used, the following preliminary steps are necessary (46): A mixture of 500 g. of fresh baker's yeast (starch-free) and 5 l. of distilled water is heated in flowing steam for 2 hr., then autoclaved at 15 lb. pressure for 15 min. The yeast is allowed to settle out. The comparatively clear supernatant liquid is filtered, then the heavier sediment. The combined filtrates are evaporated under reduced pressure to a volume of 125 cc. (Solution 1).

If autolyzed yeast is used, 50 g. of this material are suspended uniformly in a little water and diluted to 125 cc. (Solution 2).

If dehydrated yeast extract is used (this practice is most common), 25 g. of this material are dissolved in water and diluted to 125 cc. (Solution 3).

To 125 cc. of Solution 1, 2, or 3 above, is added 125 cc. of an aqueous solution containing 38 g. of lead subacetate. The precipitate is filtered out and discarded.[4] Ammonium hydroxide is added to the filtrate to produce a pH of about 10. The heavy precipitate is again filtered out with suction and discarded. Diatomaceous earth (*e.g.*, Super Cel) aids both filtrations. Glacial acetic acid is added until the solution is slightly acid (pH 6.5), and the excess lead precipitated with H_2S. After filtration, additional H_2S is bubbled through the filtrate to confirm the absence of lead. The solution is boiled gently to remove excess H_2S, and diluted with distilled water to 250 cc. The final solution contains solids equivalent to about 10% of yeast extract,[5] and is stored under toluene in the refrigerator. This treatment quantitatively removes riboflavin and folic acid from the yeast extract; so far as is known, none of the other vitamins is affected.

c. Cystine Solution. One g. of cystine is dissolved in 4 or 5 cc. of 20% HCl and the solution diluted to 1,000 cc. with distilled water.

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[4] If desired, this filtration step may be omitted and ammonia added directly.

[5] Some workers (*e.g.*, 94) prefer to use more dilute solutions than those recommended above. If desired, the same weights of starting materials can be dissolved in twice as much distilled water, and the treatment carried out as described above. The final solution then is diluted to 500 cc., and twice as much used in preparation of the double strength basal medium (10 cc. instead of 5 cc./250 cc. of medium; see p. 347).

d. Inorganic Salt Solutions. Solution A contains 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 dissolved in 250 cc. of distilled water. Solution B contains 10 g. of $MgSO_4.7H_2O$, 0.5 g. NaCl, 0.5 g. $FeSO_4.7H_2O$, and 0.5 g. $MnSO_4.4H_2O$, dissolved in 250 cc. of distilled water.

e. Glucose—Anhydrous, chemically pure glucose is used. Glucose monohydrate is also satisfactory, if used in equivalent amounts.

f. Photolyzed Yeast Extract (for Modified Medium of Greene and Black (90)). Six g. of Bacto yeast extract are dissolved in 150 cc. of distilled water, adjusted to pH 10.5 with NaOH, and exposed to direct light in a 10 in. crystallizing dish. The light source consists of two Mazda bulbs, totalling 500 watts, each at a distance of about 8 in. from the solution.[6] The temperature of the solution is kept below 50° C. by cooling with a current of air. After 10-15 hr. exposure, the solution is neutralized with HCl, diluted to 150 cc., and stored under toluene in the refrigerator.

g. Standard Riboflavin Solution. Dissolve an accurately weighed amount (10-50 mg.) of pure riboflavin in hot 0.02 *N* acetic acid (60-300 cc. of acid are required, depending upon the weight of riboflavin taken) and dilute with 0.02 *N* acid to a volume which contains 100 γ of riboflavin /cc. This stock solution is stored under toluene in the refrigerator in a brown glass bottle. A fresh solution should be prepared each month. This stock solution is further diluted (1 cc. to 1000 cc.) with distilled water to prepare the standard solution (0.1 γ of riboflavin/cc.) actually used in the assay. This latter solution is not stable on storage, and is prepared fresh just before each assay.

(4) Preparation of Basal Medium Stock Solution

To prepare 250 cc. of basal medium stock solution mix the ingredients as follows, adjust the pH to 6.6-6.8 with NaOH, and dilute with water to 250 cc.

a. Alkali-Treated Peptone Solution	50 cc.
b. Lead Acetate-Treated Yeast Extract Solution	5 cc.
c. Cystine Solution	50 cc.
d. Inorganic Salt Solutions	
A	2.5 cc.
B	2.5 cc.
e. Anhydrous Glucose	5.0 gm.

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[6] Other light sources may be used; a 3000 watt carbon arc at 8 in. destroyed the riboflavin in 1-2 hr.

This is twice the concentration of the final medium; dilution to the final concentration occurs when the various test solutions are added (see "Assay Procedure").[7]

(5) *Stock Culture and Inoculum*

Cultures of the assay organism, *Lactobacillus casei*[8] are carried as stabs in the manner discussed earlier (pp. 332). The U.S.P. and AOAC procedures use medium "a" or "b" of Table II, and transfer the stock cultures at weekly intervals.

a. Inoculum Medium. Fifty cc.[9] of the basal medium stock solution, above, 50 cc. of water, and 10 γ of riboflavin (*e.g.*, 0.1 cc. of the standard riboflavin solution) are mixed, dispensed in 10 cc. quantities to a series of test tubes, plugged with cotton, autoclaved at 15 lb. pressure for 15 min., and stored in a refrigerator until used.

b. Preparation of Inoculum. A transfer is made from a stab of the stock culture to a tube of inoculum medium. This is incubated at 37° C. for 16-24 hr. before use. A turbid culture should result. Just before use, the culture is shaken to suspend sedimented organisms. One drop of this suspension may be used directly for inoculation of each assay tube.[10]

(6) *Assay Procedure*

The assay is most conveniently carried out in standard size (16 or 18 × 150 mm.), lipless test-tubes, supported in a metal rack. To one series of tubes, the standard riboflavin solution (0.1 γ/cc.) is added in amounts as follow: 0, 0.5, 1.0, 1.5, 2.0, and 2.5 cc., corresponding to 0, 0.05, 0.10, 0.15, 0.20, and 0.25 γ of riboflavin per tube. Each level may be set up in duplicate or triplicate. An extract of each material to be assayed, prepared as described earlier, is similarly added to a series of tubes in amounts estimated to supply amounts of riboflavin which fall within the range 0.05-0.20 γ of riboflavin. At least 4 different concentration levels which fall within this range should be used, with duplicate tubes at each level. The volume of the extract added must be kept at or below 5 cc. All tubes are now diluted, where necessary, to 5 cc. with distilled water, and 5 cc. of the basal medium stock solution added to each tube. The tubes are shaken gently to facilitate mixing, plugged with nonabsorbent cotton,[11] and autoclaved at 15 lb. pressure for 10-15 min. After cooling to body heat or below, each tube is inoculated with one drop of the previously prepared inoculum suspension. All assay tubes are then incubated at 37° C. for 72 hr.

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[7] Where sample potency is to be estimated statistically, Wood (95) recommends adding sufficient riboflavin to the basal medium to give a concentration of 0.03 γ/5 cc. Since this affects the response in all tubes equally, the estimation of potency is unbiased. He thereby obtains linear dosage-response curves which greatly facilitate the statistical calculation of potency.

[8] Cultures of this organism may be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington, D. C., as number 7469.

[9] The quantities mentioned are illustrative only. More or less medium is prepared according to requirements.

[10] The USP and AOAC procedures recommend that the cells of the inoculum be thrown out by centrifugation, the supernatant liquid decanted, and the cells resuspended in 10 cc. of sterile 0.9% NaCl solution. One drop of this suspension is then added per assay tube. This precaution, introduced into microbiological assay technique to prevent carry-over of interfering quantities of the essential vitamin from inoculum to assay tube, is superfluous if the inoculum medium described above is used. One drop of the inoculum medium would supply, at most, only 0.005 γ of riboflavin, an amount insufficient to produce any significant response.

(7) Determination of Response

a. Acidimetric. The contents of each tube are transferred to a suitable container. The tube is rinsed once with distilled water, which is added to the titration vessel. The contents of this vessel are titrated with approximately $N/10$ NaOH [12] to a constant end-point in the region pH 6.8-7.0. Bromthymol blue is a suitable indicator, or electrometric titration may be used. McQuarrie and Konen (96) have described a rapid and convenient method for electrometric titration of the assay tubes.[13] Acidimetric measurement of response has been most widely used, and is recommended for most purposes.

b. Turbidimetric. Although, where results are desired quickly, turbidimetric measurements can be made after as few as 18 hr. incubation, more accurate results are obtained after longer periods of time (24 hr. or more) when growth has proceeded as far as possible (*cf.* p. 330). For determinations of comparative turbidities, photoelectric colorimeters are most commonly used. The cells of each culture are suspended by shaking; any air bubbles arising from this are allowed to rise and break, then the reading is taken. The procedure is not applicable where the amount of samples used significantly alters the color of the medium, or where turbidity not due to cells arises during the incubation period. Such instances are, however, rare. Where comparisons have been made, closely comparable results are obtained from these assays, whether acidimetric or turbidimetric measurements are made (27, 51, 8).

[11] Aluminum or glass caps may be used with considerable saving in time.

[12] The exact normality of the alkali used is of no consequence.

[13] Measurement of the pH of each tube has been suggested (97, 98) as a substitute for titration. This method is considerably more rapid than titration, and should give accurate results if a sensitive and stable pH meter is used.

(8) Calculation of Results

A standard dose-response curve for riboflavin is prepared by plotting the average of the titration values (or, if turbidimetric measurement was used, galvanometer readings or per cent transmission, *etc.*) found at each level of the riboflavin standard against the amount of riboflavin present. The riboflavin content of the sample is determined by interpolating the response to the known amounts of the test solution onto this standard curve. Since several concentrations of the test solution were used, several values will be obtained from different levels on the standard curves. Any values which show less than about 0.03 or more than about 0.20 γ of riboflavin per tube should be discarded, since observations at the two extremes of the curve are less reproducible than those in the range cited. The riboflavin content per cc. of the test solution is now calculated for each of the duplicate sets of tubes, and the riboflavin content of the sample calculated from the average of these values. Values obtained from at least three sets of duplicate tubes should be thus averaged, and the values obtained from each set should not vary by more than ± 10% from the average. If fewer than three sets of tubes yield values which fall on the usable portion of the standard curve, or if fewer than three sets of tubes give values within ± 10% of the average value, the assay is considered unreliable, and should be repeated.

Statistical methods have been recently developed for computing the results of this assay. These are discussed briefly below (pp. 353) and are given in detail in the Statistical Chapter (Vol. II). Although, in accordance with the method cited, duplicate tubes at each concentration level of standard and sample are recommended above, it should be emphasized that, when such duplicates are prepared at the same time and handled together in all subsequent steps, their agreement gives little information as to the true precision of the assay. For a full discussion of this point see Vol. II.

(9) "Drifts" and Their Possible Causes

Sometimes the computed assay values show a steady "drift," either increasing or decreasing with the concentration of the sample. The above procedure may then lead to a false estimate of potency, even though agreement between individual concentration levels is within ± 10% (99) of the average. For a reliable estimate of potency under these conditions, the sample must be reassayed under conditions which eliminate the drift. Whether the magnitude of drift is sufficient to warrant reassay under changed conditions can often be determined by application of a modified method of calculation ((99), and see also p. 353).

In the riboflavin assay, and in some other methods, serious drifts have been largely eliminated by adoption of special extraction methods, and are now encountered infrequently. In general microbiological assay work, however, such drifts occur frequently, and are one of the most troublesome phenomena encountered. They are due to substances in the sample other than the free vitamin which stimulate (or inhibit) growth of the test organism to a variable extent at different concentration levels. In some instances, such drifts can be duplicated by assay of known substances or mixtures of substances. Three general cases have been recognized. These may best be illustrated by examples. (a) A substance chemically unrelated to the vitamin, although unable to promote growth in the absence of the vitamin, may stimulate response of the test organism to suboptimal levels of it. The effect of free fatty acids on the response of *L. casei* to riboflavin (76, 82, 83, 84, 85, 86) is an example. Depending upon the concentration, the fatty acid may stimulate or inhibit the growth response to riboflavin. At levels ordinarily encountered in samples, stimulation occurs, but the per cent stimulation varies with the amount of fatty substance and with the amount of riboflavin, so that a drift in assay values is obtained at increasing levels of sample. This is usually, but not always, in the direction of decreasing values at high levels of sample. (b) Substances related chemically to the vitamin may partially or fully replace it for the test organism, but the dose-response curve to the vitamin and the related compound may be entirely dissimilar, so that the ratio of growth-promoting activity of analogue to that of vitamin is not constant at various concentration levels. Drift therefore results. The activity of "hydroxypantothenic acid" (100) is an example. This substance was 20% as active for *L. casei* as pantothenic acid when compared at low dosage levels, but only 12% as active when compared at higher levels; with *S. faecalis* R the corresponding figures were 23% and 2.4%. Similar results are obtained with *L. casei* if the activity of a lower homologue of riboflavin (7-methyl-9-(D-1′-ribityl)-isoalloxazine) is compared with that of riboflavin (89). Numerous other examples might be cited from compounds like the above analogues, which do not occur naturally. That the same phenomenon may result from the presence of combined forms of a naturally-occurring growth-factor in the extracts assayed is shown by the results of Bird and Robbins (101). They showed that the activity for *L. casei* of partial enzymatic hydrolyzates of vitamin B_c conjugate (pteroylhexaglutamylglutamic acid (102)) in terms of free vitamin B_c (folic acid, pteroylglutamic acid) varied greatly, increasing as the dosage level was increased. The activity of glutamic acid for *L. arabinosus* in terms of glutamine varies similarly on certain media (103). (c) Substances physiologically related to the vitamin, in that they may replace it for growth, may occur naturally, and interfere in the same way as described under (b) above. For example, thymine, though not related to folic acid in any obvious manner, replaces the latter for some microorganisms, and its presence in natural extracts may interfere with assays for folic acid (104).

Two general procedures are valuable for eliminating drift. Supplementation of the basal medium with an excess of the interfering substance, or vitamin-free extracts containing it, can sometimes be used in case (a) above, but not in cases (b) or (c). Modification of extraction procedures to avoid the presence of interfering materials is applicable in all cases.

(10) Specificity, Reliability and Precision of the Method

Riboflavin occurs naturally in three known forms: (a) free (b) as riboflavin phosphate and (c) as flavin adenine dinucleotide, the latter two often "bound" more or less tightly to protein. When uncombined with protein, each of these forms is reported to have growth-promoting activity for *L. casei* equivalent to its riboflavin content. This is advantageous, since they are also available to animals, and should be included in any estimate of the vitamin potency of a foodstuff.

These two combined forms of riboflavin, and certain chemical analogues of the vitamin that do not occur naturally (89), are the only known substances which permit growth of *L. casei* in the absence of free riboflavin. Many other compounds (*e.g.*, heavy metals, NaCl, ammonium salts, fatty acids, *etc.*) added to the medium in sufficient amounts, may stimulate or depress the response of the test organism to suboptimal amounts of riboflavin (*cf.* 76, 94, 82, 83, 84, 85). Only a very few of these, however, occur in natural materials in amounts sufficiently high to influence the assay adversely. Chief among these are lipoidal substances such as the free fatty acids, whose effects have been discussed above, and which are removed from the sample prior to assay by filtration or extraction with fat solvents at pH 4.5. In the assay of urines from riboflavin-deficient animals, urea may be present in interferingly large amounts, but a modified method of calculating results is claimed to eliminate the error (17).

In the hands of experienced operators, repeated assays of single samples give results which vary not more than ± 10-15% from an average figure (*e.g.*, 105). Collaborative studies (*e.g.*, 70) often show much larger variations, but these cannot logically be attributed to the estimate of potency *per se*, since similar variation occurs when physical methods, where the final estimate is much more precise, are used. Oser

(106) has called attention to the weakness of collaborative studies in this respect. In view of the widespread acceptance of the figure, ± 10%, for the precision of these assays, it is surprising to find almost no investigations where an adequate experimental basis for setting such a figure has been laid.

The accuracy of the method is, perhaps, best demonstrated by comparative analyses on the same samples by different methods. Emett *et al.* (105) used rat growth, fluorometric estimation, and microbiological assay, and found good agreement of values for a wide variety of samples. Similar agreement of these three methods applied to a mixed human diet was reported by Strong and Carpenter (76). McCrae *et al.* (107) determined the riboflavin content of food served in Royal Air Force messes by a rat-growth and microbiological procedures, and found the results in good agreement, although results obtained by rat assay tended to be slightly higher than those obtained microbiologically. An extensive comparison of the microbiological and fluorometric methods as they apply to milk products was made by Daniel and Norris (79). Agreement was excellent. Statistical analysis of 284 individual riboflavin values obtained by the two methods showed no significant difference between them. Of the 142 different samples analyzed, 82% showed less than 5% variation in results obtained by the two methods.

Results such as these, in the absence of contradictory reports, demonstrate the accuracy and reliability of the microbiological procedure, and indicate that the values found represent the true riboflavin content of the substances assayed.

(11) Application of Statistical Procedures to Microbiological Assay

In the microbiological assay of riboflavin as carried out above, the dose-response curve is usually linear over the major portion of the assay range. Where such a linear relationship exists, calculation of the potency of the sample from the ratio of slopes of the dose-response curves of standard and sample (99), rather than use of the single curve technique described above, is a superior procedure for some purposes. Where no interfering materials are present, the two methods yield the same results. In cases where, by the usual procedure, a slight "drift" in values is apparent, the slope-ratio method may give a considerably different value. As pointed out earlier, the only satisfactory way to obtain a trustworthy value on such samples is to reassay them under conditions where no drift occurs. However, under these conditions, the slope-ratio method gives a closer approximation to the true value than the single curve technique and also gives a valuable check on whether the magnitude of the drift is sufficient to require reassay.

By modifying the procedural details of the assay and employing a modified slope-ratio method for calculation, it is possible to use the microbiological data themselves to determine the variance and standard error of the result. For certain comparative studies, such techniques are invaluable. Their application is just beginning, however, and no standard procedures can be recommended as yet. For a detailed discussion of the principles involved and their application to microbiological assay, reference should be made to the chapter in Vol. II by Dr. C. I. Bliss.

At present, linear curves are usually obtained in the riboflavin assay and frequently obtained in assays for nicotinic acid, but are not obtained in most other microbiological assays. Application of these statistical methods is, therefore, possible in only a few cases. It should, perhaps, be reemphasized that an assay which gives unreliable results upon application of the single-curve method of calculation will not suddenly give accurate results merely through application of statistically sound procedures. The virtue of the latter methods is simply that the precision of the results can be ascertained from the data themselves.

Despite the fact that they are not generally applicable at present, some of the points brought out by these statistical studies are worth emphasizing because of their bearing on present procedures. For example, current practice calls for many more points to be determined on the standard curve than on the sample curve. Where the standard curve is at all regular and reproducible, this procedure is of doubtful value. The final estimate of potency is limited in its accuracy by the precision of measurement of that preparation, whether standard or unknown, which is measured with the fewest valid observations. Current procedures usually recommend duplicate or even triplicate points at each assay level, all prepared, inoculated, incubated, and titrated in nonrandom order. This procedure is statistically unsound, and investigation has shown that, under these conditions, the agreement between replicate tubes is much closer than when they are prepared, *etc.*, in random order. Only in the latter case do they reflect the true precision of the assay (unpublished data and personal communication from Dr. Bliss).

(12) Condensed Summary of Assay Methods for Riboflavin

a. Method of Snell and Strong as Modified in Detail by Collaborative Study (27, 46, 76). Assay organism—*Lactobacillus casei* ATCC 7469. Carried by weekly transfer in Medium "a" or "b," Table II.

Basal medium—Medium 2, Table IIIa.

Inoculum medium—5 cc. of basal medium + 1 γ of riboflavin and 5 cc. of water per tube.

Inoculum—Incubated 16-24 hr. at 37° C. One drop of the undiluted inoculum culture is used to inoculate each assay tube.

Concentration of riboflavin for standard curves—0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.50 γ of riboflavin. Samples are added at levels containing amounts of riboflavin within this range. Dilute to 5 cc. and add 5 cc. of double strength basal medium. For calculating results, only values which fall between 0.05 and 0.3 γ are used.

Incubation time and temperature—72 hr. at 37° C. (titrimetric); 24-30 hr. (turbidimetric).

Measurement of response—Titration with *N*/10 alkali, or turbidimetric.

b. Method of Greene and Black (90). Assay organism—*Lactobacillus casei,* ATCC 7469. Details of carrying culture not stated, but apparently the same as in Snell and Strong procedure.

Basal medium—Medium 3, Table IIIa.

Preparation of unusual constituents—See sections a, p. 345, and f. p. 347.

Inoculum medium—Same as in Snell-Strong procedure, but with lead acetate-treated yeast extract replaced by 0.1% of photolyzed yeast extract.

Inoculum—Incubated 16-24 hr. at 37° C. Centrifuged, supernatant discarded, cells suspended in 10 cc. sterile 0.9% saline, recentrifuged, supernatant discarded, cells resuspended in 4 cc. of sterile saline. One cc. of this suspension added to 100 cc. of sterile saline, and one drop of this suspension used per assay tube (*cf.* 69, and footnote 10, p. 348).

Concentration of riboflavin for standard curve—0, 0.03, 0.06, 0.09, 0.12, and 0.15 γ of riboflavin. Samples are added at levels containing amounts of riboflavin within this range.

Incubation time and temperature—72 hr. at 37° C.

Measurement of response—Titration with *N*/10 alkali.

Comments—The medium gives greater acid production and heavier growth per unit of riboflavin than the unmodified Snell-Strong procedure, and deserves wider trial than it has received. A few comparative assays by the rat-growth method gave generally comparable, though slightly lower, results (90).

c. Method of Kornberg, Langdon and Cheldelin (109). Assay organism—*Leuconostoc mesenteroides,* ATCC 10,100. Carried as stab cultures in medium b, Table II.

Basal medium—Medium 2, Table IIIa, modified by addition of *L-asparagine* (0.1 g./100 cc. of double strength medium) and addition of only one-half the indicated amount of yeast supplement (*i.e.,* 0.1 g. instead of 0.2 g./100 cc. of double strength medium). The method of preparing the alkali-treated peptone was modified slightly (see below).

Preparation of unusual constituents of basal medium.—The alkali-treated peptone was prepared as follows. Forty g. of Bacto-Peptone (Difco) is dissolved in 250 cc. of water, and to it is added a solution of 20 g. of NaOH in 250 cc. of water. The resultant mixture is brought to a boil, then allowed to stand at 37° C. for 24-48 hr. 28 cc. of glacial acetic acid and 7 g. of anhydrous sodium acetate are added and the mixture brought to a convenient volume (667 cc. $\Bumpeq$ 60 mg. peptone/cc., or 800 cc. $\Bumpeq$ 50 mg. peptone/cc.). The solution is stored under toluene.

Inoculum medium.—2.5 cc. of basal medium + 0.05 γ of riboflavin + 2.5 cc. of water.

Inoculum—Incubated 12-24 hr. at 37° C. Centrifuged, supernatant discarded, resuspend in 0.9% NaCl solution, recentrifuge, and finally resuspend in 10 cc. of saline solution. One drop of this suspension is used for each assay tube.

Concentration of riboflavin for standard curve—0, 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008 and 0.01 γ of riboflavin/10 cc. of medium. Samples are added at levels supplying 0.001-0.008 γ of riboflavin.

Incubation time and temperature—16-18 hr. at 37° C. for turbidimetric work; 72 hr. at 37° C. for acidimetric measurement.

Measurement of response—Acidimetric or turbidimetric measurement give results of equal precision.

Comments.—The test organism used requires only one-fiftieth as much riboflavin as does *Lactobacillus casei.* This higher sensitivity permits preparation of samples for assay with greater dilution than heretofore, which is especially advantageous where small samples must be used, and where samples low in riboflavin are being assayed. With the latter samples especially, decreased amounts of extraneous materials are added with the samples, and the chances for interference from such materials are correspondingly reduced. Comparative assays of a series of materials by this procedure and by the Snell-Strong procedure gave results in excellent agreement. Less interference from fatty materials occurred with low potency samples (unenriched white flour, for example) in the present procedure, so that special precautions to remove free fatty acids were unnecessary.

This assay is as sensitive to riboflavin on the 10 cc. scale as is the ultramicro procedure of Lowry and Bessey, described below, which employs volumes of only 0.2 cc. By a corresponding reduction of volumes, the present assay procedure could be made sensitive to amounts of riboflavin much smaller than can be determined by any other procedures available (*i.e.,* 0.02-0.16 mγ). The procedure is too recent to have been widely tested as yet.

(13) *Estimation of Riboflavin on the "Ultramicro" Scale*

For determination of the distribution of riboflavin or other vitamins in minute quantities of relatively unavailable materials, it is possible to cut the scale of the customary assays by as much as 50-fold, so that they are carried out in a total volume of 0.2 cc. instead of 10 cc. In the first such attempts (110), the cultures were grown in the depression of spot plates, with fairly regular and reproducible results. More recently, Lowry and Bessey (32) have investigated in detail the factors influencing growth of *L. casei* in small volumes and have applied their findings to the microbiological assay of riboflavin.

The procedural modifications worked out for this small-scale assay should be applicable without appreciable change (other than in the basal medium) to the assay of other vitamins with *L. casei* (and, perhaps, with other lactic acid bacteria). For this reason, the procedure is given below in full. For further details and data in support of the precision of the assay, reference should be made to the original publication (32).

a. Reagents and Stock Solutions. The basal medium used is the same as that described above (Medium 2, Table IIIa), except that 0.15 *M* potassium acetate (1.5%) is substituted for the sodium acetate (0.6%). For this purpose, KOH (28 g.) replaces the NaOH (20 g.) used in preparation of the alkali-treated peptone solution 3a, (p. 345). Following neutralization with glacial acetic acid, 71 g. of anhydrous potassium acetate is added (in place of 7 g. of sodium acetate), and the solution is diluted to 800 cc.

b. Preparation of Basal Medium Stock Solution. This is prepared exactly as described in section 4 (p. 347), using the modified alkali-treated peptone solution described in a, above.

After the medium has been sterilized, and just before inoculation (see procedure below), 0.5 cc. of a 0.4% cysteine solution is added to each 10 cc. of the basal medium. A few cc. of this cysteine solution are freshly prepared before each assay, from either cysteine and water, or from cysteine hydrochloride with the calculated amount of NaOH. The solution is boiled just before use, and added to the sterile basal medium with a sterile pipette.[14]

c. Stock Cultures and Inoculum. Stock cultures are carried as in the macro procedure, with transfer at weekly intervals. Inoculum is prepared as in the macro procedure, by supplementing the basal medium stock solution (b above) with riboflavin.

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[14] It was found that, despite the substitution of CO_2 for air in this micro-scale assay, occasional assays failed. This failure was traced to use of basal media which had been autoclaved, then allowed to stand for some time in the ice box before use. Addition of a freshly prepared cysteine solution just before use always overcame this trouble. Cystine or thioglycolic acid, though helpful, were not as effective as cysteine (32).

d. Assay Procedure. The assays are carried out in serological test tubes, 6 × 50 mm. (0.75 cc.). These are cleaned by boiling briefly in half concentrated HNO_3, then boiling in distilled water.[15] Extracts of the tissues may be prepared as recommended in the macro procedure, but where only small amounts are available, the method given below is used (32).

"To the tissue sample containing 2-8 mγ of riboflavin is added 0.1 ml. of 0.1 *N* HCl, either with a 0.2 ml. graduated pipette, the end of which has been drawn out to a slender tip, or better, with a Levy-Lang constriction pipette (111). The tubes are plugged with cotton and autoclaved for 15 min. at 15 lb. pressure, with care thereafter to protect from light, especially while still hot. The weight of a few tubes before and after autoclaving should be determined to see that no significant change occurs in the volume. After cooling, exactly 0.3 ml. of 0.03 *N* NaOH is added with a slender tipped 1 ml. graduated pipette or a constriction pipette, and each tube is promptly and thoroughly mixed by twirling in the tube a slender rod with a hooked end. This leaves the solution with an excess acid concentration of about 0.002 *N*. Three 0.1 ml. aliquots are transferred to 0.75 ml. serological tubes (6 × 50 mm.). At the same time, tubes for establishing a standard curve are prepared by measuring out 0.1 ml. in triplicate of each of four standards and a blank. The standards contain 0.5, 1.0, 1.5 and 2.0 mγ of riboflavin/0.1 ml. or 0.5, 1.0, 1.5 and 2.0 γ/100 ml., and are made up in 0.002 *N* HCl. The blank is 0.002 *N* HCl.

"All the tubes are plugged with cotton and autoclaved for 15 min. at 15 lb. pressure. The whole rack should be wrapped in black cloth during autoclaving and until thoroughly cooled.

"The tubes are inoculated as suggested by Pennington *et al.* (110), by pipetting into each tube 0.1 ml. of basal medium previously inoculated with 2 drops of a washed suspension of *Lactobacillus casei* per 10 ml. The suspension of bacilli is prepared as in the regular Snell and Strong procedure.[16] The medium is added with a 1 ml. sterilized pipette, the tip of which is drawn out to a slender point and slightly bent. The volume of basal medium must be delivered with an accuracy of 2 or 3%

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[15] The acid wash is necessary to remove surface alkali from the glass, which may otherwise destroy a significant fraction of the small amount of riboflavin added for assay (32).

[16] As in the "macro" procedure, it is probably unnecessary to use a washed suspension for inoculum.

for best results. The cotton plug is replaced and each tube is mixed by tapping with the finger. Flaming of the mouth of the tube is unnecessary.

"The rack of tubes is placed in a vacuum desiccator containing damp cotton swabs[17] and the air therein replaced with CO_2[18] by alternately reducing the pressure to about 150 mm. of Hg and introducing CO_2 back to atmospheric pressure. After this procedure is repeated 4 or 5 times the pressure is left at about 700 mm. of Hg and the desiccator is placed in an incubator at 38° C. for 3 days.

"The tubes are then removed from the desiccator. A minute droplet of caprylic alcohol is added to each tube to prevent foaming and the CO_2 is blown off by carefully aerating each tube with a capillary tube of not over 0.5 mm. diameter. The bubbles should be small enough so that spattering is entirely confined to the tube. After aeration for 1 min., a 0.02 ml. drop of 0.04% bromthymol blue is added and the tube is titrated with 0.3 *N* NaOH from a 0.2 ml. Rehberg burette. Stirring is accomplished by bubbling with air. Neither the air bubbler nor the burette tip should be over 0.7 mm. in diameter. Since the indicator is soluble in caprylic alcohol, the amount of caprylic alcohol used should be kept small, and bubbling during titration should not be too prolonged. While one tube is being titrated, the next tube is being aerated, so that the preliminary aeration does not add to the time involved.

"The results are calculated from the standard curve, just as with the microbiological assay on 10 ml. However, with some tissues, the results are influenced by the presence of substances other than riboflavin in the tissue extract. The following procedure appears to obviate this difficulty in a most satisfactory manner, and should be used for highest precision if conditions will permit. Tissue of the type being analyzed is extracted, *etc.*, exactly as described above, but on a larger scale. The final extract in 0.002 *N* HCl is irradiated for 30 min. in a Pyrex tube at a distance of 3 or 4 cm. from a General Electric HB-4 mercury arc lamp to destroy the riboflavin present. With as little dilution as possible, standards are prepared from this extract containing 0, 0.5, 1.0, 1.5 and 2.0 mγ of riboflavin per 0.1 ml. To produce the standard curve, these standards are used in place of pure riboflavin solutions." (32).

e. Precision of the Method. Lowry and Bessey showed that pure riboflavin could be estimated by the above procedure with a probable mean error, expressed as per cent of the mean, of about 1%. The probable error with biological materials was greater, about 3%, for the tissues (rat cornea) to which the method was applied.

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[17] These serve to saturate the gaseous contents of the desiccator with water vapor and thus prevent evaporation from the assay tubes.

[18] Experimental results quoted in the original paper showed that considerably greater acid production per unit of added riboflavin resulted when the test was incubated in CO_2 rather than air. CO_2 was superior to N_2; O_2 was particularly deleterious. *Cf.* also the discussion, p. 336.

(14) Other Lactic Acid Bacteria Available for Assay of Riboflavin

A large number of lactic acid bacteria other than *L. casei*, ATCC 7469, are known which require riboflavin for growth. These include, among the lactobacilli, certain strains of *L. acidophilus, L. beijerinckii, L. bulgaricus, L. delbrueckii, L. gayonii, L. helveticus, L. jugert, L. lactis, L. leichmanii,* and *L. lycopersici;* and among the streptococci, numerous strains of *S. cremoris, S. faecalis, S. lactis, S. salivarius, S. thermophilus* and others (for references see (22)). The list is not exhaustive. It would be strange indeed if, among these many organisms, one or several could not be found which was more suitable for riboflavin determination than *L. casei* 7469, the present test organism. Among the lactobacilli, Campbell and Hucker (112) found one organism, identified only as No. 7993, but thought to be a strain of *L. bulgaricus,* which required riboflavin in considerably smaller amounts (⅓-⅕ as much) than the present organism, and which appeared to give reliable assay values for riboflavin. If equally suitable in other respects, such an organism would have some advantages over *L. casei,* since smaller amounts of sample (and presumably smaller amounts of possible interfering materials) are required for assay. Among the streptococci, too, cultures are known which require smaller amounts of riboflavin than does *L. casei.*[19] The very recently developed method of Kornberg, *et al.* (109), which uses a strain of *Leuconostoc mesenteroides* which requires only one-fiftieth as much riboflavin as does *L. casei,* has been described briefly above, and illustrates the advantages which may accrue from investigation of the utility of additional test organisms. Since the procedure with *L. casei* is known to give reliable results with a great variety of samples, it would appear that this method should be retained until the newer modifications have been similarly tested by widespread use.

B. NICOTINIC ACID

(1) Extraction from Natural Materials

Nicotinic acid is one of the most stable of the known vitamins (71). It is not destroyed by light, concentrated acids or alkali, or by most oxidizing agents. In pure form it can be sublimed without decomposition. Comparatively drastic procedures can, therefore, be used, where necessary, to effect its complete extraction from natural materials. For the most widely used assay organism, *L. arabinosus,* its extraction offers little difficulty. For many materials, simple extraction with hot water gives maximum values (48). Other materials, however (*e.g.,* cereal products (113, 114, 115, 116) and dehydrated foods (117)), require acid or alkaline hydrolysis for complete extraction. For general use, an acid extraction procedure has become standard.

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[19] Personal communication to the author from Dr. E. A. Zeller.

a. Recommended Procedure for Extraction with Acid (118). A convenient and accurately known weight of the finely ground or homogenized sample is suspended or dissolved in sufficient $N/1$ H_2SO_4 to give an estimated nicotinic acid content of 1 γ/cc. of mixture.[20] The mixture is heated in an autoclave at 15 lb. pressure for 15 min., cooled, adjusted with NaOH solution to pH 6.8, and diluted to an estimated nicotinic acid content of 0.1-0.2 γ/cc. Filtration is generally unnecessary, but may be carried out if desired. Aliquots of this solution are used for assay.

(2) Assay Media for Nicotinic Acid and Their Development

That certain lactic acid bacteria require nicotinic acid for growth was shown in 1938 (7, 119). By 1941, knowledge of the nutritional requirements of these organisms permitted development of a medium (No. 8, Table IV), which permitted no significant growth of *L. arabinosus* in the absence of nicotinic acid, but which supported excellent growth when nicotinic acid was added in sufficient amounts (33, 48). This medium and organism were widely used for the determination of nicotinic acid. Experience with the method has resulted in a number of minor modifications of the medium (Table IV). Isbell (39) noted that his culture of *L. arabinosus* required *p*-aminobenzoic acid, and recommended addition of this substance to the medium. The effect of this substance is observed with difficulty (50, 120), and it may not be required for all strains of the organism (41, 120), however, its addition to the medium is advisable. Krehl *et al.* (50) noted improved acid production and stability in behavior of the test organism when the glucose and acetate levels in the medium were increased. In addition to these changes, Barton-Wright (121) recommends increasing the concentration of casein hydrolyzate, and addition of xanthine and xylose. Other alterations are apparent by inspection of the table. At the risk of repetition (*cf.* p. 343), it should again be emphasized that the aim of these methods is to permit convenient and precise determinations of nicotinic acid, and not necessarily to permit the maximum possible growth and acid production by the test organism. The authors of modified methods have dealt almost solely with the latter point, on the assumption that any modification which improves acid production per unit of added vitamin will also improve precision of the assay method. Though plausible in many respects, this assumption does not hold in some instances, and should be tested experimentally by direct comparison of values (and their variation) obtained on repeated assay of identical extracts with the two different media.

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[20] For substances extremely low in nicotinic acid (< 5 γ/g. dry weight), larger volumes of extractant should be used.

TABLE IV

Basal Media for Determination of Nicotinic Acid with L. arabinosus

	Medium No.				
Constituents	8. Snell and Wright 1941 (48)	9. Krehl *et al.* 1943 (50)	10. Barton-Wright 1944 (121)	11. Collaborative USP-AOAC 1945 (118)	12. Lawrence, *et al.* 1946 (44)
	g./100 cc. of double strength medium				
Hydrolyzed casein (charcoal treated)	1.0	1.0	2.0	1.0	1.0
Glucose	2.0	4.0	4.0	4.0	4.0
Sodium acetate	1.2	4.0	4.0	2.0	4.0
L-Cystine	0.02	0.04	0.04	0.04	0.04
L-Tryptophan	0.02	0.02	0.02	0.01	0.02
L-Asparagine hydrate	—	—	—	—	0.05
Xylose	—	—	0.2	—	—
	mg./100 cc. of double strength medium				
Adenine	2.0	2.0	2.0	2.0	2.0
Guanine	2.0	2.0	2.0	2.0	2.0
Xanthine	—	—	2.0	—	—
Uracil	2.0	2.0	2.0	2.0	2.0
	γ/100 cc. of double strength medium				
Riboflavin	40	40	40	40	80
Thiamine	20	20	20	20	100
Calcium pantothenate	20	20	20	20	100
Pyridoxine	20	20	20	20	200
p-Aminobenzoic acid	—	20	20	20	50
Biotin	0.08	0.04	0.08	0.08	0.30
Inorganic salts			[b]		
Solution A [a]	1 cc.	1 cc.		1 cc.	1 cc.
Solution B [a]	1 cc.	1 cc.		1 cc.	1 cc.

[a] Salts A contain 100 mg. K_2HPO_4 and 100 mg. KH_2PO_4/cc. Salts B contain 40 mg. $MgSO_4.7H_2O$, 2.0 mg. NaCl, 2.0 mg. $FeSO_4.7H_2O$, and 2.0 mg. $MnSO_4.4H_2O$/cc.

[b] 1.0 g. NaCl, 100 mg. K_2HPO_4, 100 mg. KH_2PO_4, 40 mg. $MgSO_4.7H_2O$, 2.0 mg. $MnSO_4.4H_2O$, 0.12 mg. $FeCl_3$. In a subsequent paper (91) Barton-Wright recommends the further addition of 0.3% (0.6 g.) of ammonium sulfate.

Such inadequate comparisons as are available show no significant differences in assay values obtained with any of the media of Table IV.

Media 4 and 7, Table IIIb, are modifications of medium 8, Table IV, devised, by addition of folic acid, to permit growth of *L. casei*. They are, of course, also suitable for growth of *L. arabinosus*. By addition of riboflavin and omission of nicotinic acid they can be used for assay of nicotinic acid with either *L. casei* or *L. arabinosus*. (See section on generalized media, p. 418).

A somewhat different type of medium for assay of nicotinic acid with *L. arabinosus* was devised by Sarett *et al.* (117). By exhaustive adsorption with Lloyd's Reagent (a special adsorptive fuller's earth), nicotinic acid was removed from yeast and rice bran extracts. These, together with hydrolyzed casein and a charcoal-treated yeast extract, then serve as nitrogen source in the medium. Such treatment removes certain other vitamins also essential for *L. arabinosus*, but since these essentials are all known (48) they can be added back to the medium in pure form. The presence of these nicotinic acid-free natural extracts improves growth by supplying substances of unknown nature which, though not essential for the test organism, increase its growth in the presence of suboptimal amounts of nicotinic acid. The composition of this modified

TABLE IVb

Composition of Media for Assay of Nicotinic Acid with L. arabinosus
(Continued)

Constituent	Medium No. 13. Sarrett, Pederson and Cheldelin, 1945 (117) Amount per 100 cc. of double strength medium
Hydrolyzed casein[a] (charcoal treated)	1.0 g.
Glucose	4.0 g.
Sodium acetate	3.6 g.
Lloyd's reagent treated:	
Peptone	0.50 g.
Liver extract	0.20 g.
Yeast extract	0.20 g.
Norite treated yeast extract	0.20 g.
Cystine	.04 g.
Calcium pantothenate	20 γ
Inorganic salts[b]	
Solution A	1 cc.
Solution B	1 cc.

[a] Hydrolyzed with H_2SO_4.

[b] As in Table IV.

medium is given in Table IVb. A small number of comparative assays with media 8, 9 and 13 gave identical results (within experimental error) for the nicotinic acid content of the substances assayed. However, the authors state that the modified medium minimizes drifts obtained with certain samples on the older, semi-synthetic media.

Any of the above media can undoubtedly be used with *L. arabinosus* for the accurate assay of nicotinic acid. Because of its semi-official standing, preparation and use of Medium 11 will be described below. The detailed directions below are essentially those of a widely used, collaborative procedure (118), and are designed for use by laboratory technicians. The procedures used for preparation of stock solutions, *etc.*, illustrate conditions under which the constituents of the medium are stable and the assay conveniently carried out. Many research workers will find it convenient to modify and simplify them to fit their own problems and working conditions. It is an easy matter to determine whether such modification affects adversely the reliability of the assay result. For those who wish to use medium 13, the preparation of the special supplements necessary and a summarized procedure are also described.

(3) Reagents and Stock Solutions

a. Acid Hydrolyzed-Casein Solution.[21] Mix 100 g. of purified casein (*e.g.*, Labco "vitamin-free" casein is suitable for direct use) with 500 cc. of approximately 20% HCl and reflux the mixture for 16-24 hr. Remove excess HCl from the mixture by distillation under reduced pressure until a thick paste remains. Dissolve this residue in distilled water, adjust the solution to pH 3.5 (± 0.1) with a NaOH solution, and dilute with water to 1,000 cc. To this solution add 20 g. of activated charcoal (*e.g.*, Norit

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[21] In the original method of Snell and Wright (48), and in the modified method of Barton-Wright (121), H_2SO_4-hydrolyzed casein is used. HCl-hydrolyzed casein is equally, if not more, satisfactory, and is easier to prepare. It contributes comparatively large (and uncontrolled) amounts of NaCl to the medium, however, so that inclusion of small extra quantities of this salt with the mineral mixture is superfluous. Where media of known salt content are desired, H_2SO_4-hydrolyzed casein is useful. It is prepared as follows: 100 g. of purified casein are mixed with 500 cc. of 25% (by volume) H_2SO_4. The mixture is autoclaved for 10 hr. at 15 lb. pressure. The solution is diluted to *ca.* 2 l., and a hot concentrated solution of $Ba(OH)_2$ added until the mixture is almost neutral. The $BaSO_4$ is allowed to settle, the supernatant solution siphoned off, and the sludge filtered dry with suction and washed. Use of generous quantities of a filter aid (*e.g.*, Super-Cel) is helpful. All liquid fractions are combined, and barium ion exactly removed with dilute H_2SO_4. $BaSO_4$ is removed by filtration, and the filtrate concentrated under reduced pressure to 1000 cc. Charcoal treatment, as in the procedure above, is usually necessary to remove the last traces of nicotinic acid.

A or Darco G-60), stir for 1 hr. and filter.[22] The filtrate (1 cc. equivalent to 100 mg. casein) should be light yellow or colorless. The solution is stored under toluene in a refrigerator.

b. Cystine-Tryptophan Solution. Suspend 4 g. of L-cystine and 1 g. of L-tryptophan (or 2 g. of DL-tryptophan) in 200-300 cc. of distilled water, heat to 70-80° C., and add 20% HCl dropwise with stirring until the solids are just dissolved. Cool and dilute with distilled water to 1,000 cc. Store in the refrigerator.

c. Adenine-Guanine-Uracil Solution. Dissolve 0.1 g. each of adenine sulfate, guanine hydrochloride, and uracil with the aid of heat in 5 cc. of 20% HCl, and dilute to 100 cc.

d. Riboflavin-Thiamine-Biotin Solution. Prepare a solution containing in each cc. 20 γ of riboflavin, 10 γ of thiamine hydrochloride, and 0.04 γ of biotin by dissolving the pure crystalline vitamins in 0.02 *N* acetic acid. Store the solution in the refrigerator under toluene.

e. p-Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Solution. Prepare a solution containing 10 γ of each of these vitamins per cc. by dissolving pure calcium *d*-pantothenate, *p*-aminobenzoic acid, and pyridoxine hydrochloride in neutral 25% alcohol. Store the solution in a refrigerator.

f. Inorganic Salt Solutions A and B (See (d) p. 347).

g. Standard Nicotinic Acid Solution. Dissolve an accurately weighed sample of pure nicotinic acid in alcohol, and dilute with 25% alcohol to a concentration of 100 γ/cc. Store this solution in a refrigerator. The working standard, prepared fresh for each assay, is prepared from this stock solution by allowing a portion to come to room temperature, then diluting 1 cc. with distilled water to 1,000 cc. (final concentration, 0.1 γ/cc.).

h. Special Supplements for Use with Medium 13, Table IVb (117). (1) Lloyd's reagent-treated peptone, liver and yeast mixture. 25 g. of Bacto-peptone, 10 g. of liver extract (Lederle or Wilson) and 10 g. of yeast extract (Difco) are autoclaved 15 min. with 400 cc. of 1 *N* NaOH solution. The solution is cooled, acidified to pH 0.8-1.0 with conc. HCl, diluted to 500 cc. and 45 g. of Lloyd's reagent added. The mixture is agitated 20 min. and centrifuged. The supernatant liquid is decanted, the pH readjusted to 0.8-1.0, and the adsorption is repeated twice. Eighteen g. of K_2HPO_4 are added and the pH is adjusted to 6.6. The extract is steamed, cooled, and filtered. Each cc. is thus equivalent to

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[22] The charcoal-treatment serves to remove traces of nicotinic acid present in the hydrolyzate. The amount of charcoal which can be used is limited, since excessive amounts will adsorb certain essential amino acids and thus prevent growth in the final medium.

50 mg. of peptone, 20 mg. of liver extract, and 20 mg. of yeast extract. The solution is stored under toluene in a refrigerator.

(2) Norit-treated yeast extract. Ten g. of yeast extract (Difco) are steamed 10 min. in 200 cc. of water. After cooling, the pH is adjusted to 1.5 with conc. HCl and 10 g. of activated charcoal (Norit) are added. The mixture is shaken 20 min., filtered, the pH is readjusted to 1.5, and the adsorption is repeated. The filtrate is neutralized to pH 6.6, steamed, cooled, and filtered. One cc. of the final solution contains residual solids from 50 mg. of the original yeast extract. The extract is also free of pantothenic acid (cf. 52).

(4) Preparation of Basal Medium Stock Solution, (Medium No. 11, Table IV)

To prepare the basal medium stock solution mix the ingredients as follows, adjust the pH to 6.6-6.8 with NaOH, and dilute with water to 500 cc. This gives a double strength medium; dilution to final concentration is made when the various test samples are added (see "Assay Procedure" below).

a. Acid-hydrolyzed casein solution	50 cc.
b. Cystine-tryptophan solution	50 cc.
c. Adenine-guanine-uracil solution	10 cc.
d. Riboflavin-thiamine-biotin solution	10 cc.
e. *p*-Aminobenzoic acid-calcium pantothenate-pyridoxine hydrochloride solution	10 cc.
f. Inorganic salt solutions	
Solution A	5 cc.
Solution B	5 cc.
g. Glucose, anhydrous	20 gm.
h. Sodium acetate, anhydrous	10 gm.

(5) Stock Culture and Inoculum

Cultures of the assay organism, *Lactobacillus arabinosus* 17-5,[23] are carried in stabs in the manner discussed earlier (pp. 335). The AOAC-USP collaborative procedure (118) uses medium "c" of Table II, with 2% of yeast extract instead of 1%, and recommends transfer at weekly intervals.

a. Inoculum Medium. To each of a series of tubes containing 5 cc. of the stock solution of basal medium add 5 cc. of distilled water containing 1 γ of nicotinic acid. Plug the tubes with non-absorbent cotton, sterilize in an autoclave at 15 lb. steam pressure (121° C.) for 20 min., and store in a cold room or refrigerator until needed.

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[23] Cultures of this organism may be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington, D. C., as number 8014.

b. Preparation of Inoculum. The day before the assay is prepared, a transfer is made from a stab of the stock culture to a tube of the inoculum medium. This culture is incubated for 16-24 hr. at a temperature of 30-37° C. The culture thus obtained is shaken to suspend the cells uniformly, and then may be used directly as inoculum.

(6) Assay Procedure

It is convenient to carry out the assay in lipless test tubes, 16 or 18 × 150 mm., individually supported in a metal rack. To one series of such tubes, the freshly diluted standard nicotinic acid solution (0.1 γ/cc.) is added as follows with duplicate tubes at each level: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 cc. (0 to 0.5 γ), respectively. An extract of each material to be assayed, prepared as described earlier, is similarly added to a series of tubes in amounts estimated to supply amounts of nicotinic acid which fall within the range 0.05 to 0.45 γ. At least 4 different concentration levels which fall within this range should be used, with duplicate tubes at each level. The volume of extract added is kept at or below 5.0 cc. All tubes are now diluted with distilled water to 5 cc., and 5.0 cc. of the basal medium stock solution added to each tube. The tubes are shaken gently to promote mixing, plugged with cotton or covered with aluminum or glass caps, and autoclaved at 15 lb. pressure for 10-15 min. After cooling to 37° C., or less, each tube is inoculated with 1 drop of the inoculum suspension. The racks of tubes are incubated at any selected temperature between 30 and 37° C. (but held as constant as possible, preferably within ± 0.5°) for 72 hr.

(7) Determination of Response

The acid produced at each concentration level is measured by direct titration.[24] See section 7a, p. 349.

(8) Calculation of Results

A standard curve is plotted and results calculated in the way described for riboflavin (See section 8; and also sections 9 and 11 under "Riboflavin").

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[24] If the basal medium is sufficiently free of nicotinic acid, turbidity measurements may also be used.

(9) Specificity, Reliability, and Precision of the Method

A number of substances closely related to nicotinic acid occur naturally. Of these, nicotinamide, nicotinuric acid, cozymase (48), and the nicotinamide nucleoside from partially hydrolyzed cozymase (122) are equally active on the molar basis for *L. arabinosus.* Trigonelline (48), arecoline, and N-methylnicotinamide (122) are inactive. The activity of nipecotic acid is so slight (0.0001 that of nicotinic acid) that it might result from contamination of the preparation with traces of nicotinic acid; in any case, its activity is negligible (123). Coramine is inactive, but active following hydrolysis (124). Except for coramine, which is active for the dog, and nicotinuric acid, where the data are conflicting, activities of these compounds for *L. arabinosus* parallel those reported for animals (125). Neither of these substances is generally distributed. A large number of other compounds chemically related to nicotinic acid have been tested for activity with both dogs and microorganisms with generally concurrent results.

An unidentified compound, which gives rise to nicotinic acid on hydrolysis with alkali or acid, occurs in cereal grains (113, 114, 116). Concentrates of the substance show low activity for *L. arabinosus* before hydrolysis, with much higher activity following hydrolysis (115). The substance is probably utilized by the dog, but not by the chick (122). It appears to be an ester of nicotinic acid with an acidic, water-soluble compound (115).

Trigonelline can be converted to nicotinic acid under some conditions by alkali treatment (114, 126, 127); however, increases due to this conversion during alkaline hydrolysis of food samples have not been observed (116, 114).

It is general experience that the method for nicotinic acid is among the most reliable and least troublesome of the microbiological methods. Aside from internal evidence such as obtention of theoretical recoveries of nicotinic acid, agreement of assay values at increasing levels of concentration, *etc.*, the best evidence for accuracy comes from comparative results of chemical and microbiological assays on similar or identical samples. In making such comparisons, it should be remembered that chemical methods for nicotinic acid underwent extensive modifications during the years 1937-41, and that prior to the latter date, even the best methods often gave high results. Several comparisons of chemical and microbiological methods made since that time have shown excellent agreement between the two methods. Such comparisons have been made with meat products (128, 129), cereal products (116), and mixed foods (114, 121, 129, 130). Collaborative studies on cereal products (116) showed good agreement between average values obtained by several workers by microbiological and chemical procedures, but a rather wide variation between individual workers by both methods (*cf.* (106) for a discussion of this phenomenon). An especially convincing comparison was reported by Daniel and Norris (131). With 31 varieties of beans, peas, soybeans, peanuts and lentils, these authors found a maximum difference of only 7.7% between nicotinic acid values obtained by the microbiological method of Snell and Wright (48) and the chemical method of Dann and Handler (132); the average difference was only 3%. This is smaller than the experimental error of either method. A sole report that the microbiological method gave erroneous results on cereal products (133) was not confirmed by two other groups (121, 50) working with similar samples. The accuracy of the microbiological method is thus well established. For further detailed discussion of this assay method see (134).

(10) Condensed Summary of Assay Methods for Nicotinic Acid Employing Lactic Acid Bacteria

a. Collaborative AOAC-USP Methods ((118)—A Modification of the Method of Snell and Wright (48)).

Assay organism—*Lactobacillus arabinosus* 17-5 ATCC No. 8014. Carried as stab cultures by weekly transfer in Medium "c," Table II, with 1% additional yeast extract.

Basal medium—Medium 11, Table IV. Initial pH of medium should be 6.5-7.0.

Inoculum medium—5 cc. of basal medium + 1 γ nicotinic acid and 5 cc. of water per tube.

Inoculum—Grown 18-24 hr. at 30-37° C. One drop of undiluted suspension is used per assay tube.

Concentrations of nicotinic acid for standard curve—0, 0.5, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 γ/10 cc. of basal medium. Samples added at levels which contain corresponding amounts of nicotinic acid.

Incubation time and temperature—72 hr. at any temperature between 30-37° C., but maintained constant to within ± 0.5°.

Measurement of response—Titration with $N/10$ alkali.

b. Method of Sarett, Pederson and Cheldelin (117).

Assay organism—*Lactobacillus arabinosus* 17-5 ATCC No. 8014. Carried as stab cultures by monthly transfer in Medium "b," Table II.

Basal medium—Medium 13, Table IVb. Initial pH of medium should be 6.5-7.0.

Preparation of unusual constituents—See section *h*, p. 365.

Inoculum medium—5 cc. of Basal medium + 1 γ nicotinic acid, 5 mg. liver extract (Lederle or Wilson), 5 mg. yeast extract and 5 cc. of water per tube.

Inoculum—Grown 16-24 hr. at 37° C. (preferable 18 hr.). 1 cc. of inoculum culture added to 15 cc. of sterile, 0.9% NaCl solution; one drop of this diluted suspension is used per assay tube.

Concentrations of nicotinic acid for standard curve—0 to 0.5 γ/10 cc. Samples added to furnish corresponding levels of nicotinic acid.

Incubation time and temperature—40-72 hr. at 37° C.

Measurement of response—Titration with *N*/10 alkali.

Comment—The medium is stated to permit somewhat greater growth per unit of added nicotinic acid than do the semi-synthetic media previously used, and to give superior results with samples which show "drift" on other media.

c. Method of Johnson (37).

Assay organism—*Leuconostoc mesenteroides*, ATCC No. 9135. The organism is carried by biweekly transfer in a medium containing yeast extract, 1%; milk peptone (Difco), 1%; glucose, 1%; and agar, 1.5%.

Basal medium—Medium 14, Table V. Initial pH adjusted to 6.5-7.0.

Inoculum medium—5 cc. of basal medium + 1 γ of nicotinic acid and 5 cc. of water per tube.

Inoculum—Grown 24 hr. at 30° C. Centrifuge out the cells, and resuspend them in 10 cc. of sterile 0.9% saline. One drop of this suspension is used per assay tube.

Concentrations of nicotinic acid for standard curve—0 to 3.0 γ/10 cc.; assay range about 0.2-2.0 γ of nicotinic acid. Samples are added to furnish these levels of nicotinic acid.

Incubation time and temperature—72 hr. at 30° C.

Measurement of response—Titration with *N*/10 alkali. 13-14 cc. of *N*/10 acid are produced in response to excess nicotinic acid.

Comment—The test organism (*Leuconostoc mesenteroides*) utilizes free nicotinic acid, but in contrast to *L. arabinosus*, cannot utilize nicotinamide or nicotinuric acid. The method, therefore, permits determination of free nicotinic acid in samples. Nicotinamide is quantitatively hydrolyzed to nicotinic acid by heating for 1 hr. at 121° C. (15 lb. pressure in the autoclave) with 0.6 *N* H_2SO_4. This procedure does not hydrolyze nicotinuric acid, so that the nicotinamide content of a sample can be obtained by assay before and after hydrolysis. Total nicotinic acid (*i.e.*, nicotinic acid, nicotinamide, and nicotinuric acid) can be determined with *L. arabinosus;* the difference between this figure and that obtained with *Leuconostoc mesenteroides* following hydrolysis with 0.6

N H_2SO_4 represents nicotinuric acid. Comparative assay figures obtained with both organisms (135) failed to show significant amounts of nicotinuric acid in sweat or urine. The behavior of the organism toward coenzymes I and II has not been described. The method has not been widely used. Minor modifications of this method and further details of its application to natural materials for the differential determination of nicotinic acid and nicotinamide are given by Krehl *et al.* (136).

(11) Other Lactic Acid Bacteria Available for Assay of Nicotinic Acid

All of the lactic acid bacteria whose nutritive requirements are known require nicotinic acid for growth (22, 33). For special purposes, use of other organisms for the assay might prove advantageous. Because of the satisfactory results obtained with *L. arabinosus*, few other organisms have been investigated.

TABLE V

Composition of Medium for Assay of Nicotinic Acid with Leuconostoc mesenteroides

Constituent	Medium No. 14, Johnson 1945 (37) Amount/100 cc. of double strength medium
Hydrolyzed casein (vitamin free)	1.0 g.
Glucose	6.0 g.
Sodium acetate	4.0 g.
Cystine	2.0 mg.
L-Tryptophan	1.0 mg.
Inorganic salts	
Solution A[a]	1.0 cc.
Solution B[a]	1.0 cc.
Riboflavin	50 γ
Thiamine hydrochloride	50 γ
Calcium pantothenate	50 γ
Biotin	1.0 γ
Pyridoxal	10 γ
p-Aminobenzoic acid	10 γ
Adenine	2.0 mg.
Guanine	2.0 mg.
Xanthine	2.0 mg.
Asparagine	5.0 mg.

[a] As in Medium 8, Table IV. Solution A contains 100 mg. K_2HPO_4 and 100 mg. KH_2PO_4/cc.; solution B contains 40 mg. $MgSO_4.7H_2O$, 2.0 mg. NaCl, 2.0 mg. $MnSO_4.4H_2O$, and 2.0 mg. $FeSO_4.7H_2O$/cc.

C. THIAMINE

(1) *Extraction from Natural Materials*

Thiamine occurs naturally in the free form and as the pyrophosphate, cocarboxylase. The monophosphate, derived from cocarboxylase by hydrolytic removal of one phosphate residue, probably also occurs in many samples. All of these forms may also occur bound more or less firmly to protein. For complete extraction of thiamine, hydrolysis is therefore required. The vitamin is very easily destroyed by heating in alkaline or neutral solution, but is quite stable in strongly acid solutions (71). For liberation from natural materials, hydrolysis with acid or with phosphorolytic enzymes, or with each consecutively has become standard practice. The procedure outlined below is essentially that recommended in the U.S.P. thiochrome assay (46). The last step, consisting in adsorption on and elution from a base exchange silicate (Decalso) is unnecessary when microbiological assays are used, and is therefore omitted.

a. Recommended Procedure for Extraction of Thiamine (46). A convenient and accurately known weight of the finely ground or homogenized sample is suspended or dissolved in at least 15 times its weight of 0.1 *N* H_2SO_4. The mixture is digested on a steam bath with frequent mixing for 30 min. The liquid must remain distinctly acid during the digestion: if the pH rises above 1.5, additional dilute H_2SO_4 is added. After heating, the mixture is cooled, and the pH adjusted to between 4 and 4.5 by addition of a concentrated solution (2 *N* or greater) of sodium acetate. This is the optimum pH for digestion with phosphorolytic enzymes. A 10% solution of an enzyme preparation high in diastatic and phosphorolytic enzymes[25] is prepared in distilled water. For each 10 γ of thiamine estimated to be present in the sample used, 1 cc. of this solution is added. The mixture is incubated at 45-50° C. for 3 hr., or over night at 37° C.[26] It is then adjusted to pH 6.5 to 6.6, diluted to an estimated thiamine content of 0.1-0.2 γ/cc., filtered if cloudy, and suitable aliquots used for assay.

b. Alternate Procedure for Extractions of Thiamine (35).[27] One g. of the finely ground or blended sample is digested at 37° C. under benzene for 16-20 hr. with 20 mg. each of papain and takadiastase in 40 cc.

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[25] Takadiastase, clarase, Mylase-P, or Polidase-S are suitable commercial enzyme preparations for this purpose.

[26] If over-night incubation is used, the mixture must be protected with benzene or toluene from bacterial action.

[27] This procedure has not been widely tested by collaborative assay, as has the preceding method of extraction.

of 0.5% acetate buffer, pH 4.5 (78). The digest is then heated in a steamer for 10 min., diluted to 50 cc., filtered through Celite, and the pH adjusted to 6.5-6.6. Suitably diluted aliquots are then added to the assay tubes.

(2) Assay Media for Thiamine and Their Development

Cultures of lactic acid bacteria which have been most widely used as assay organisms (*e.g., L. arabinosus, L. casei, S. faecalis* R) do not require thiamine as a growth factor. It was only after semisynthetic media such as No. 4 or 6 (Table IIIb), or No. 8 (Table IV), had been developed and used with other organisms that cultures of lactic acid bacteria which required addition of thiamine to such media for growth were found. Similar semisynthetic media were then readily developed for use in assay of thiamine. Since thiamine is very readily destroyed by standing (or especially on heating) at room temperature and can be removed from natural materials by adsorption, thiamine-deficient media based upon crude extracts are also easily developed. Media of both types have been used, and are shown in Table VI.

(3) Condensed Summary of Assay Methods for Thiamine Employing Lactic Acid Bacteria

a. Method of Niven and Smiley (68).

Assay organism—*Streptococcus salivarius* S 20B.[28] Carried as stab cultures by transfer at 6 week intervals in a meat infusion agar containing 1% tryptone, 0.1% glucose, 0.2% K_2HPO_4, 1.5% agar, and excess $CaCO_3$.

Basal medium—Medium No. 15, Table VI. Initial pH adjusted to 7.4.

Preparation of unusual ingredients of medium—Thiamine-free yeast extract. Six g. of Difco Bacto-yeast extract are dissolved in 200 cc. of water and autoclaved (15 lb.) for 15 min. The pH is adjusted to 3.0, 10 g. of fuller's earth added, and the mixture shaken for 30 min. The filtrate is adjusted to pH 1.0, reautoclaved for 15 min., cooled, a second 10 g. portion of fuller's earth added, and the mixture shaken over night. After filtering, 1.5 g. of K_2HPO_4 are added to the filtrate, which is then adjusted to pH 7.4 and autoclaved again for 15 min. The resulting precipitate is filtered out, and the volume adjusted to 200 cc. This solution is sterilized in conveniently sized lots, and stored until used.

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[28] Although one strain of *S. salivarius* was used in developing the assay method, any strain is believed suitable, since 20 strains examined all had the same growth-factor requirements (137). The organisms are easily detected and isolated from throat smears of normal individuals (138).

Inoculum medium—5 cc. of basal medium + 4 cc. of water. Sterilize by autoclaving, cool, then add 1 cc. of a sterile solution containing 10 mγ (.01 γ) of thiamine hydrochloride/cc. Several tubes of medium may be

TABLE VI

Composition of Media for Assay of Thiamine with Lactic Acid Bacteria

For *Streptococcus salivarius*

Medium No. 15, Niven and Smiley, 1943 (68)

Constituent	Amount/100 cc. of double-strength medium
Casein hydrolyzate [a]	1.0 g.
Thiamine-free yeast extract	0.6 g.
Glucose	2.0 g.
Potassium phosphate buffer (0.4 *M*, pH 7.0)	20 cc.
Salt solution [b]	2.0 cc.
Uracil	1.0 mg.
Nicotinic acid	100 γ
Riboflavin	100 γ
Calcium pantothenate	100 γ
Biotin (methyl ester)	0.1 γ
For *Lactobacillus fermenti* 36 Medium No. 16, Sarett and Cheldelin, 1944 (35)	
Alkali-treated peptone (Plus sodium acetate) [c]	2.0 g.
Casein hydrolyzate [a]	0.5 g.
Glucose	4.0 g.
Sodium acetate	1.2 g.
Cystine	20 mg.
Adenine sulfate	2.0 mg.
Guanine hydrochloride	2.0 mg.
Uracil	2.0 mg.
Salts A [d]	1.0 cc.
Salts B [d]	1.0 cc.
Riboflavin, calcium pantothenate, *p*-aminobenzoic acid, nicotinic acid, pyridoxine hydrochloride	20 γ each
Biotin	0.08 γ
Folic acid [e]	0.015 γ

[a] A 10% acid-hydrolyzed, vitamin-free casein, prepared with H_2SO_4 as described on p. 364. Traces of thiamine, if present, are removed with Norit at pH 3.0. Ten (35) or 20 (68) g. of Norit are used/100 g. of casein.

[b] The stock solution of salts contains 10 g. of NaCl, 0.8 g. $MgSO_4.7H_2O$, 40 mg. $FeSO_4.7H_2O$, and 12 mg. $MnCl_2$ in 100 cc. of distilled water.

[c] Prepared as originally described by Snell and Strong (27), but without exposure to light (see p. 345).

[d] See footnote to Table IV for composition of these salt solutions.

[e] Recalculated in terms of pure folic acid (pteroylglutamic acid), assuming this to have a potency of 137,000.

made up at once but care should be taken to steam the medium for several minutes before use to insure prompt growth.

Inoculum—Grown 24 hr. (or until "good" growth occurs) at 37° C. Five-tenths cc. of the resulting suspension is mixed with 10 cc. of sterile 0.9% NaCl solution, and one drop of this diluted suspension used per assay tube.

Concentration of thiamine for standard curve—0, 0.1, 0.2, 0.4, 0.7, 1.0 and 2.0 $m\gamma/10$ cc. Samples are added to furnish thiamine at several levels within this range. Since thiamine is destroyed by autoclaving at the initial pH of the medium, sterile solutions of both standard and samples are added aseptically after the sample tubes have been autoclaved.[29] In preparing the sample tubes before autoclaving, distilled water is added in volumes such that addition of the sterile standard and sample after autoclaving will make the total volume to 10 cc.

Sterilization—15 min. at 15 lb. pressure.

Incubation time and temperature—37° C. for 24 hr.

Measurement of response—Turbidimetric most satisfactory, although good results are reported from titration with 0.05 N NaOH.

Comment—The thiazole and pyrimidine moieties of thiamine are inactive for the test organism, both alone and in combination, at levels up to 10 $\gamma/10$ cc. of medium. Cocarboxylase is approximately 40% more active than thiamine on a molar basis; the monophosphate of thiamine is also probably more active than thiamine itself. This makes complete hydrolysis of the cocarboxylase of natural materials to thiamine obligatory before accurate values can be obtained.

Because standard and samples must be sterilized separately and added aseptically to previously autoclaved sample tubes, the method is considerably more tedious than the previously presented assays for riboflavin and nicotinic acid. From published results, it does not appear to have been widely used, so that few data bearing on its applicability and accuracy are available. Limited data presented in the original publication appear satisfactory; recoveries of added thiamine of from 100 to 124% were reported.

Some of the disadvantages of this method are avoided in the method using *L. fermenti* as test organism, described below.

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[29] A sterile standard solution of thiamine hydrochloride (100γ/cc.) is prepared by dissolving 10 mg. of the vitamin in 100 cc. of 0.1 M acetate buffer, pH 4.5, and autoclaving for 15 min. at 15 lb. pressure. Aliquots are diluted appropriately with sterile distilled water just before use. Samples must also be sterilized in a similar fashion before addition to the sterilized assay tubes. The authors report somewhat superior results if the glucose, too, is autoclaved as a separate solution and added aseptically after sterilizing the assay tubes.

b. Method of Sarett and Cheldelin (139, 35).

Assay organism—*Lactobacillus fermenti* 36, ATCC No. 9338. Carried as stab cultures by monthly transfer in medium "d," Table II, supplemented with 10 γ of thiamine per tube.

Basal medium—Medium 16, Table VI. Initial pH of medium, 6.5.

Inoculum medium—5 cc. of basal medium, 10 γ thiamine, 5.0 mg. of Difco yeast extract, with distilled water to 10 cc. Sterilized by steaming or autoclaving as usual before use.

Inoculum—Incubated 16-24 hr., but not longer, at 37° C. Cells are centrifuged out, resuspended in 10 cc. of sterile saline, and 1 drop of this suspension further diluted with 25 cc. of sterile saline. One drop of this dilute suspension is used to inoculate each assay tube.

Concentration of thiamine for standard curve—0, 5, 10, 15, 20, 30, 40 and 50 mγ (0-0.05 γ) of thiamine hydrochloride per 10 cc. of diluted medium. Samples are usually run at four different levels ranging from 5 to 40 mγ of thiamine per assay tube (10 cc.).

Sterilization—Plugged tubes containing standard and samples are heated in flowing steam (100° C.) for 15 min. At the pH of the medium (6.5) this procedure avoids destruction of thiamine, and is adequate to avoid contamination during the short assay period used.

Incubation time and temperature—16-18 hr. at 37° C.

Measurement of response—Turbidimetric, with photoelectric turbidimeter. A 5400 Å light filter is recommended.

Comment—The procedure is convenient; separate sterilization of samples and standard is not required. Available data indicate that it is accurate. Reported recoveries of added thiamine range from 90 to 108%; values obtained check values obtained by applying the thiochrome procedure to the same materials. Pyrimidine and thiazole moieties of thiamine show no growth-promoting activity during an 18 hr. incubation period. With longer incubation periods (*e.g.*, 20-24 hr. and longer), some activity of these products is apparent. Cocarboxylase is about 30% more active than an equimolar amount of thiamine; the test organism thus resembles *S. salivarius* (68) in specificity of response. The method is a recent one, and has not yet been subjected to the test of widespread use. When carried out as originally described (35), the test organism sometimes develops the ability to synthesize thiamine at a low rate, which is reflected in standard curves with excessively high blank values. This tendency is effectively prevented by inclusion of the high level of thiamine recommended above in the media for carrying stock cultures and growing inocula (139).

(4) Other Lactic Acid Bacteria Available for Assay of Thiamine

In view of the complex nutritional requirements of lactic acid bacteria, it is interesting that only a few of these organisms are so far known which require thiamine. As noted above, all strains of *S. salivarius* and many strains of *L. fermenti* require the vitamin. In addition to these organisms, Niven (38) found that thiamine was required for many strains of *S. lactis*. Some strains of *Leuco. mesenteroides* also require the vitamin, and use of this organism for its determination has been suggested (36).

D. PANTOTHENIC ACID

(1) Extraction from Natural Materials

That pantothenic acid occurs naturally in a "bound" form, unavailable for growth of yeast, and that liberation of additional pantothenic acid occurs during autolysis of tissues, was noted shortly after the discovery of this vitamin (140). After pantothenic acid had been identified as a vitamin for the chick (141, 142), it became evident from comparative assays that, even after autolysis, only a fraction of the total pantothenic acid of tissues (as indicated by chick assay) was available to microorganisms (*cf.* 143). As pantothenic acid is readily destroyed by treatment with acid or alkali (71, 144), enzymatic procedures for releasing the vitamin have been used. A variety of enzymes have proved somewhat effective, but commercial enzyme preparations high in amylolytic and phosphorolytic activity (takadiastase, clarase, Mylase P, *etc.*) are most satisfactory (143, 78, 145). Such enzyme preparations also contain peptidases and other miscellaneous enzymes. It is not known at present which component (or components) of the mixture is effective in liberating pantothenic acid. They have been used both alone (143, 145) and in conjunction with a proteolytic enzyme, papain (78), for release of the vitamin. The most thorough investigation of use of these enzymes for release of pantothenic acid is that of Ives and Strong (145). The procedure recommended by them is given in (*a*) below.

Although this procedure is much more effective than autolysis alone in releasing pantothenic acid from natural materials, the values obtained on extracts prepared in this manner are still considerably below those obtained by chick assay of similar tissues (146). One reason for the discrepancy became apparent when it was found by Lipmann and coworkers that coenzyme A (a coenzyme necessary for certain acetylations) was a pantothenic acid complex (147), that it was widely distributed in nature (148, 149), and that it was not broken down to a microbiologically available form by enzymes such as clarase and papain which had previously been used for liberation of pantothenic acid from tissues (147). These workers showed that a mixture of a liver enzyme and an alkaline phosphatase did release pantothenic acid from coenzyme A (147, 150) and have given details of their procedures (150).

The utility of this mixture of enzymes in releasing pantothenic acid from its bound forms in natural materials has been compared with that of "mylase P" (procedure *a*, below) by Neilands and Strong (151). In many materials, treatment with mylase P released only 20-30% as much pantothenic acid as did treatment with the mixed enzymes, and in no case did it release as much pantothenic acid as the mixed enzymes. The latter procedure, which is the best one for application to natural materials thus far available is given in (*b*) below. No comparison of the values obtained by chick assay and by microbiological assay following the new digestion procedure is available, and it is, therefore, still not certain that all of the pantothenic acid of natural materials is being released. It is apparent, however, that the new procedure permits a much closer approximation to complete release of pantothenic acid than do any previously used methods.

The response of both *L. casei* and *L. arabinosus*—the two organisms most widely used for assay—to pantothenic acid is markedly affected by the presence of fatty acids, lecithin, *etc.*, in the sample. As with riboflavin, growth in the presence of suboptimal quantities of pantothenic acid may be greatly stimulated, suppressed, or unaffected by these lipoidal materials, depending upon their concentration in the sample. For a documented discussion of these effects, reference should be made to the section dealing with extraction of riboflavin. The measures used to prevent these effects are the same as with riboflavin assay: (a) filtration of the digested sample through paper at pH 4.5-4.8, and (b) extraction of the digested sample with fat solvents preliminary to assay. Except with samples which are extraordinarily rich in lipoidal materials, application of the first of these precautionary measures is usually sufficient.

a. Procedure for Digestion with Mylase P (145). An accurately known quantity of the finely ground or homogenized sample estimated to contain between 3 and 10 γ of pantothenic acid is suspended in 50 cc. of water. The suspension is adjusted to pH 6.8-7.0 and autoclaved for 15 min. at 15 lb. pressure. It is then cooled to room temperature, and an amount of "Mylase P"[30] equal to one-tenth the estimated dry weight of the sample is added to the flask. The contents are buffered with 2 cc.

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[30] "Mylase P" is obtainable from the Wallerstein Laboratories, New York.

of 2.5 *M* sodium acetate solution, and the pH lowered to 4.8 with HCl. The mixture is then layered with toluene, corked, and incubated for 12-24 hr. at 50° C. At the end of this time, the sample is diluted to 100 cc. with distilled water. The pH is checked, and readjusted, if necessary, to pH 4.8. The sample is then filtered through a fluted Whatman No. 40 paper, repeatedly if necessary, until a clear filtrate is obtained. Suitable aliquots of this filtrate are used for assay.

b. Procedure for Digestion with a Mixture of Alkaline Phosphatase and Liver Enzyme (Tentatively Recommended Procedure) (151). Both enzymes must be prepared from natural materials. The alkaline phosphatase is prepared from calf intestine by the method of Schmidt and Thannhauser (152).

This procedure involves collection of the mucosa from calf intestine (the first two yards from the pylorus), dilution and stirring at pH 9.0, digestion with trypsin, precipitation of the phosphatase with 0.9 saturated $(NH_4)_2SO_4$, solution, filtration, and reprecipitation with 0.8 saturated $(NH_4)_2SO_4$, dialysis against ammonia-ammonium acetate buffer of pH 8.8, adsorption of impurities with $Al(OH)_3$, removal of contaminating trypsin by adsorption with kaolin, precipitation of the active enzyme from solution with ice-cold acetone, solution of the precipitate and reprecipitation with acetone, following which the enzyme is dissolved in water, dialyzed against ammonium acetate buffer, and stored in the refrigerator as an aqueous solution which is stable for months. The original publication must be consulted for details of this well standardized and easily duplicable procedure. The potency of the final solution obtained may be variable; that used by Neilands and Strong (151) contained 1150 phosphatase units/mg. of nitrogen and was kept at a concentration of 1484 units/cc.

Preparation of the acetone-dried liver which serves as a source of the liver enzyme is described in detail by Kaplan and Lipmann (148). Neilands and Strong found the more readily available chicken livers to be as good or better as a source material than the pigeon livers used by Kaplan and Lipmann. Livers from freshly killed chickens or pigeons are cooled, minced, and homogenized with 20 volumes of *cold* acetone in a Waring blendor. The precipitate is collected, washed with acetone and ether, dried, and kept as the dry powder. When desired for use, a portion of this powder is extracted with 10 times its weight of ice cold 0.02 *M* $NaHCO_3$ and centrifuged. The enzyme is labile in solution at room temperatures.

No standardized procedure for use of the two enzymes with a variety of natural materials can yet be given. Neilands and Strong (151) recommend use of the minimum amount of sample in order to keep the enzyme

TABLE VII

Composition of Media for Assay of Pantothenic Acid with L. casei

Constituent	17. Pennington, Snell and Williams, 1940 (47)	18. Strong, Feeney and Earle, 1941 (45)	19. Neal and Strong, 1943 (53)	20. Ives, Zepplin, Ames, Strong and Elvehjem, 1945 (154)
	Medium No.			
	Amount/100 cc. of double strength medium			
Peptone, alkali-treated	1.0 g.	1.0 g.	1.0 g.	1.0 g.
Yeast extract, alkali-treated	0.2 g.	—	—	—
Yeast extract, charcoal-treated	—	1.0 g. (≎)	1.0 g. (≎)	—
Vitab,[a] charcoal-treated	—	—	1.5 g. (≎)	—
Casein, acid-hydrolyzed	0.4 g.	—	—	—
Glucose	2.0 g.	2.0 g.	2.0 g.	2.0 g.
Sodium acetate[b]	1.2 g.	1.2 g.	4.0 g.	4.0 g.
Cystine	20 mg.	20 mg.	20 mg.	20 mg.
Asparagine	—	50 mg.	50 mg.	50 mg.
Glutamic acid	—	—	50 mg.	50 mg.
Riboflavin	20 γ	20 γ	20 γ	20 γ
Nicotinic acid, *p*-aminobenzoic acid, thiamine chloride, pyridoxine hydrochloride	—	—	—	20 γ each
Folic acid[c]	—	—	—	.015 γ[c]
Inorganic salts				
Solution A[d]	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
Solution B[d]	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.

[a] A commercial rice-bran concentrate in a molasses vehicle; produced by National Oil Products Company.

[b] 1.2 g. of the total sodium acetate is supplied by the alkali-treated peptone when prepared by the recommended procedure.

[c] Recalculated on the basis that pure folic acid has a "potency" of 137,000 (92, 15, 93).

[d] As in Medium 8, Table IV.

concentration in excess. They used 1-40 mg. of the homogenized sample, 0.05 cc. of the chicken or pigeon liver enzyme, 0.2 cc. of the intestinal phosphatase, 0.05 cc. of 0.1 *M* $NaHCO_3$ solution and distilled water to a volume of 1 cc. The tubes were incubated at 37° C. for 4 hr., diluted, and assayed. If interfering fatty materials are present, the pH should be readjusted to 4.5-4.8 and the solutions filtered as in (*a*) above before assay. A "blank" sample containing only the enzyme preparations is always run, and the pantothenic acid content found is subtracted from that found for the digested samples.

(2) Assay Media for Pantothenic Acid and Their Development

In 1937 it was shown that all lactic acid bacteria tested required for growth minute amounts of an acidic, acid- and alkali-labile substance (6), which had been purified considerably from natural materials. The deficient test medium used contained alkali-treated peptone, glucose, sodium acetate, inorganic salts and riboflavin (Medium 1, Table IIIa, with riboflavin added and pantothenic acid omitted). Following identification of this growth factor as pantothenic acid (7, 119), and synthesis of this vitamin (153), the medium was reinvestigated as a possible basal medium for determination of pantothenic acid in crude natural extracts. For this application, it was found advisable to enrich the medium, and this has been accomplished in various ways (*cf.* Media 17 and 18, Table VII). Following elucidation of the other nutritional essentials for lactic acid bacteria it has also become possible to employ semisynthetic media for the determination of pantothenic acid. Thus, Medium 21, Table VIII, represents a slight modification of Medium 8, Table IV, originally presented for assay of nicotinic acid.

Only 2 lactic acid bacteria, *L. casei* and *L. arabinosus,* have been widely studied as assay organisms for pantothenic acid. *L. casei* was used in the early fractionation work with pantothenic acid (6) and its use carried over into the early quantitative methods. Subsequent work has shown that the nutritional requirements of *L. arabinosus* are considerably simpler than those of *L. casei* (33), and this organism has been preferred in more recently studied assay methods. A recent questionnaire (154) indicates that methods employing *L. arabinosus* are in much wider use than the earlier methods employing *L. casei* simply because experience has indicated that better results (*i.e.,* more reproducible results, with fewer erratic samples, and less "drift") are obtained with this organism. Because *L. arabinosus* has simpler requirements for growth than does *L. casei,* it is evident that any of the assay media recommended for *L. casei* might be used for *L. arabinosus* with satisfactory results; the converse, however, is not usually true.

(3) Condensed Summary of Assay Methods for Pantothenic Acid Employing Lactic Acid Bacteria

a. Method of Pennington, Snell and Williams (47).

Assay organism—*Lactobacillus casei,* ATCC No. 7469. Carried as stab culture by monthly transfer in Medium "b," Table II.

Basal medium—Medium 17, Table VII. Initial pH of medium, 6.6-6.8.

Preparation of unusual ingredients of medium—

(1) Alkali-treated peptone—Prepared exactly as described for riboflavin assay (p. 345), except that exposure to light is unnecessary.

(2) Alkali-treated yeast extract—20 g. of Difco yeast extract in 200 cc. of 0.5 *N* NaOH is autoclaved at 15 lb. pressure for 30 min. The solution is neutralized with glacial acetic acid, autoclaved an additional 10 min. and filtered. The solution is adjusted to 200 cc. (100 mg./cc.), and preserved under toluene.

(3) Acid-hydrolyzed casein—Technical casein is sufficient, and is hydrolyzed with H_2SO_4 as described in footnote to p. 364, or with HCl as described p. 364.

Inoculum medium—5 cc. of basal medium, "an excess" (0.5-1.0 γ) of calcium pantothenate, and 5 cc. of distilled water. Sterilized by autoclaving as usual before use.

Inoculum—Incubated 24 hr. at 37° C. Cells are then centrifuged out, resuspended in an equal volume of sterile 0.9% NaCl solution, and 1 drop of the resulting heavy suspension used to inoculate each assay tube.

Concentration of calcium pantothenate for standard curve—0, .015, .03, .04, .05, .07, .10, .15, and .20 γ of calcium pantothenate/10 cc. of diluted medium. Samples are run at 4-6 levels estimated to fall within this range.

Sterilization—Autoclaved 15 min. at 15 lb. pressure.

Incubation time and temperature—24 hr. at 37° C. for turbidimetric measurement, 3 days at 37° C. for acidimetric.

Measurement of response—Turbidimetric or acidimetric.

Comment—Occasional workers have reported failure of the treatment with alkali to remove all pantothenic acid from the yeast extract. This may be remedied by increasing slightly the time of autoclaving. Supplementation of the medium with a portion of the extract to be assayed, which has been freed of pantothenic acid by acid or alkaline hydrolysis, is an effective procedure for eliminating erratic results produced by occasional samples.

This medium is the earliest one proposed for assay of pantothenic acid in natural materials. The author prefers later methods using *L. arabinosus;* alternatively, *L. arabinosus* may be used in the above procedure in place of *L. casei.*

b. Method of Strong, Feeney and Earle (45).

Assay organism—*Lactobacillus casei* ATCC No. 7469. Carried as stab cultures by monthly transfer in Medium "a," Table II.

Basal medium—Medium 18, Table VII. Initial pH of medium, 6.8-7.0.

Preparation of unusual ingredients of medium—

(1) Alkali-treated peptone—Prepared as described for riboflavin assay (p. 345), except that exposure to light is omitted.

(2) Charcoal-treated yeast extract—25 g. of whole, autolyzed baker's yeast[31] are suspended in 500 cc. of water. Conc. HCl is added to pH 1.5. 25 g. of activated charcoal (Norit A) are added and the mixture is stirred 20 min. at room temperature. The charcoal is then removed by centrifugation or filtration. A small amount of charcoal appearing in the filtrate is disregarded. The filtrate is readjusted to pH 1.5, and the adsorption repeated as before. The filtrate from this second treatment is adjusted to pH 7.0 with NaOH, filtered to remove any charcoal present and diluted to 1000 cc. (1 cc. $\Bumpeq$ 25 mg. of original autolyzed yeast). It is stored under toluene in the refrigerator. Once started, the preparation should be carried to completion as rapidly as convenient, since holding the solution at pH 1.5 for long periods of time is thought to be deleterious.

Inoculum medium—5 cc. of basal medium, 0.5 γ calcium pantothenate, and distilled water to 10 cc. Sterilized by autoclaving as usual and stored until use.

Inoculum—Incubated 24-48 hr. at 37° C. The cells are centrifuged out, resuspended in 10 cc. of sterile 0.9% NaCl solution. One cc. of this suspension is diluted with 10 cc. of sterile saline, and 1 drop of this latter suspension used to inoculate each assay tube.

Concentration of calcium pantothenate for standard curve—0, .03, .05, .075, .10, .125, .15 γ/10 cc. of diluted medium. Samples are run at levels which fall within this range; preferably, at least 3 levels should fall within the range 0.03-0.12 γ. Duplicate tubes are used throughout.

Sterilization—Autoclaved 15 min. at 15 lb. pressure.

Incubation time and temperature—72-84 hr. at 37° C.

Measurement of response—Titration with 0.1 *N* NaOH.

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[31] **Obtainable from Difco Laboratories, Inc., Detroit, Michigan.**

Comment—This procedure has been modified by further study; the modifications are incorporated in methods of Neal and Strong (53) and Ives *et al.* (154), given below.

c. Method of Neal and Strong (53).

Assay organism—*Lactobacillus casei* ATCC No. 7469. Carried as stab cultures by weekly transfer in Medium "a," Table II.

Basal Medium—Medium 19, Table VII. Initial pH of medium 6.8-7.0.

Preparation of unusual ingredients of medium—

(1) NaOH treated peptone: see method of Strong, Feeney and Earle, above.

(2) Charcoal-treated yeast extract: prepared as described in preceding method except that Darco G-60 was used in place of Norit A, and the adsorption was carried out in a volume of 240 cc. After the second charcoal treatment the filtrate was neutralized with solid NaOH and diluted to 250 cc. (1 cc. $\approx$ 100 mg. of original autolyzed yeast).

(3) Charcoal-treated Vitab—75 g. of Vitab are dissolved in 500 cc. of water, the pH adjusted to 1.2-1.5 with conc. HCl, and the mixture stirred with 50 g. activated charcoal for 20 min. at room temperature. The charcoal is filtered off, the pH readjusted to 1.2-1.5, and the adsorption repeated. The filtrate is adjusted to pH 6.8 with NaOH, filtered again, and diluted with distilled water to 1000 cc. (1 cc. $\approx$ 75 mg. of Vitab). It may be preserved under toluene in the refrigerator.

Inoculum medium—5 cc. basal medium, 1 γ calcium pantothenate, and 5 cc. distilled water per tube. Sterilized by autoclaving as usual.

Inoculum—Prepared as in the preceding method of Strong, Feeney and Earle.

Concentration of calcium pantothenate for standard curve—0, .01, .02, .04, .06, .08 and .10 γ/10 cc. of diluted medium. Samples assayed at several levels to furnish pantothenic acid within this range. Standard curve is linear within assay range.

Sterilization—Autoclaved 15 min. at 15 lb. pressure.

Incubation time and temperature—72-84 hr. at 37° C.

Measurement of response—Titration with 0.1 *N* NaOH.

Comment—The addition of glutamic acid and the Vitab supplement increases the acid produced by *L. casei* per unit of added calcium pantothenate. Approximately 0.08 γ of vitamin produce the same response (9 cc. of 0.1 *N* acid) in this medium as did 0.14 γ of vitamin in the previous medium of Strong, Feeney and Earle.

d. Method of Ives, Zepplin, Ames, Strong and Elvehjem (154). The procedure is exactly the same as that of Neal and Strong, given above, except that Medium 20 is used throughout in place of Medium 19. Yeast and vitab supplements are thus eliminated; the sensitivity of the organism to calcium pantothenate remains the same, and the medium is reported to give excellent results with no drift, when the samples are prepared by the enzymatic digestion procedures of Ives and Strong (145) (see under "Pantothenic Acid. Extraction from Natural Materials" above).

Comment—This medium has not been widely tested. A recent survey (155), carried out before Medium 20 became available revealed that 10 of 11 analysts preferred *L. arabinosus* to *L. casei* as an assay organism for pantothenic acid. Media suggested for use with the former organism are discussed below. *L. arabinosus* has simpler nutritional requirements than does *L. casei;* any of the above media, suitable for assay with *L. casei,* could also be used with *L. arabinosus.*

e. Method of Skeggs and Wright (156).

Assay organism—*Lactobacillus arabinosus* ATCC No. 8014. Carried as stab culture by monthly transfer in Medium "b," Table II.

Basal medium—Medium 21, Table VIII. Initial pH of medium 6.6-6.8.

Preparation of unusual ingredients of medium—

(1) Hydrolyzed casein, charcoal-treated. Prepared as for nicotinic acid assay (see p. 364).

Inoculum medium—1% Difco yeast extract, 1% glucose. Tubed in 10 cc. lots, sterilized as usual, and stored until used.

Inoculum—Incubated 24 hr. at 33° C.; cells centrifuged out and resuspended in sterile physiological saline. A portion of this heavy suspension is further diluted with sterile saline to yield a faintly turbid suspension; one drop of which is used to inoculate each assay tube.

Concentration of calcium pantothenate for standard curve—Prepare tubes containing 0, .01, .02, .04, .06, .08, .1, and 0.2 γ calcium pantothenate/10 cc. of final medium. The assay range is from about 0.01-0.08 γ.

Sterilization—Autoclaved 15 min. at 15 lb. pressure.

Incubation time and temperature—3 days for acidimetric measurements; turbidity measurements can be made after 18 hr.

Measurement of response—Titration with 0.1 *N* NaOH routinely. Successful results obtained turbidimetrically after 18 hr. are cited in the original paper.

Comment—The assay medium is a modification of the Snell-Wright medium for nicotinic acid (Medium 8, Table 4), with glucose, sodium acetate, and vitamins increased in concentration. A survey of labora-

TABLE VIII

Composition of Media for Assay of Pantothenic Acid with L. arabinosus

Constituent	Medium No. 21. Skeggs and Wright, 1944 (156)	Medium No. 22. Hoag, Sarett and Cheldelin, 1945 (157)
	Amount per 100 cc. of double strength medium	
Peptone, alkali-treated	—	1.0 g.
Casein, acid-hydrolyzed, charcoal-treated	1.0 g.	0.4 g.
Yeast extract, charcoal-treated	—	0.2 g.
Vitab, charcoal-treated	—	1.5 g.
Glucose	4.0 g.	4.0 g.
Sodium acetate [a]	1.2 g.	3.6 g.
Cystine	20 mg.	20 mg.
Tryptophan	20 mg.	—
Uracil	1.0 mg.	—
Adenine	1.0 mg.	—
Guanine	1.0 mg.	—
Xanthine	1.0 mg.	—
p-Aminobenzoic acid	20 γ	—
Nicotinic acid	200 γ	—
Pyridoxine hydrochloride	400 γ	—
Riboflavin	200 γ	20 γ
Thiamine chloride	200 γ	—
Biotin	0.5 γ	—
Inorganic Salts		
Solution A [b]	1.0 cc.	1.0 cc.
Solution B [b]	1.0 cc.	1.0 cc.

[a] One g. of alkali-treated peptone, when prepared according to the recommended procedure, supplies 1.2 g. of sodium acetate.

[b] As in Medium 8, Table IV.

tories conducted in 1945 (155) indicated this to be the most widely preferred procedure for determining pantothenic acid of the methods available at that time. The medium is not suitable for growth of *Lactobacillus casei.*

f. Method of Hoag, Sarett and Cheldelin (157).

Assay organism—*Lactobacillus arabinosus* ATCC No. 8014, carried as stab cultures by monthly transfer in Medium "b," Table II.

Basal medium—Medium 22, Table VIII. Initial pH of medium, 6.8.

Preparation of unusual ingredients of medium—

(1) Hydrolyzed casein—Prepared as described under "Assay method for Nicotinic Acid," (p. 364).

(2) Charcoal-treated yeast extract—Prepared as described by Neal and Strong (see under "Method of Neal and Strong," p. 384 above).
(3) Charcoal-treated Vitab—See under "Method of Neal and Strong, p. 384 above.

Inoculum medium—5 cc. of basal medium, 1 γ calcium pantothenate, 5 mg. yeast extract (Difco), 5 mg. of liver extract (Lederle) and water to 10 cc. per inoculum tube. Tubes are autoclaved as usual and stored until use.

Inoculum—Incubated at 37° C. for 16-24 hr. One cc. of this suspension is diluted in 15 cc. of sterile 0.9% NaCl solution. One drop of the resulting dilute suspension is used for each assay tube.

Concentration of calcium pantothenate for standard curve—0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2 γ/10 cc. of dilute medium. 0-0.08 γ is the preferred assay range.

Sterilization—Not specified; apparently the customary 15 min. at 15 lb. pressure.

Incubation time and temperature—37° C. for 24-72 hr. for acidimetric assay (longer periods preferred); 14-24 hr. for turbidimetric assay.

Measurement of response—Titration with 0.1 *N* alkali, or turbidimetric.

Comment—With added pantothenic acid, the medium supports excellent growth of both *L. arabinosus* and *L. casei,* and comparative assays showed the same assay figures (within experimental error) to be obtained with either organism. *L. arabinosus,* however, grows faster, and produces slightly greater amounts of acid, and is therefore preferred for assay use. The medium without charcoal-treated yeast and vitab supplements also supports excellent growth, and is very similar to a recently recommended medium, details of which are not available (158). The authors state, however, that "drifts" with certain samples are less noticeable where these supplements are included.

(4) Other Lactic Acid Bacteria Available for Assay of Pantothenic Acid

All of the lactic acid bacteria whose requirements have been investigated require pantothenic acid for growth (119, 159, 42, 38). Of these, only two, *L. casei* and *L. arabinosus,* have been used for its quantitative estimation in natural materials. It is evident that many other organisms might be used successfully for assay, either with existing media, or with additional specially developed assay media.

E. BIOTIN

(1) Extraction from Natural Materials

The earliest quantitative studies of the distribution of biotin were made by Kögl and coworkers (160, 8) by a yeast growth method. In these classical studies, which led to the first isolation of biotin (8), the existence of bound forms of the vitamin, unavailable to the test organism, apparently was not appreciated. Subsequent work showed that, from many tissues, additional biotin (or vitamin H) was liberated by autolysis (161, 162), by digestion with proteolytic enzymes (161, 78), by autoclaving at high pressures with water (161), or by hydrolysis with mineral acids (161, 163, 164). In most cases, bound biotin considerably surpasses free biotin in amounts present, so that, if figures for the total biotin content are desired, some type of hydrolysis is required. Although, in some instances, maximal values are obtained by enzymatic digestion or other procedures, acid hydrolysis is usually required for complete liberation of biotin. In a study of the necessary conditions, Lampen *et al.* (163) found it necessary to heat samples with 2-4 N H_2SO_4 for 2 hr. in the autoclave at 15 lb. pressure to effect complete release of biotin for *Clostridium acetobutylicum*. The hydrolytic treatment necessary varies somewhat with the test organism. Thus, enzymatic digests of many materials give maximal values for biotin when *L. casei* is used as the test organism, but yield lower values when *L. arabinosus* is employed. The discrepancy is eliminated by hydrolysis with 6 N H_2SO_4 at 15 lb. for 1 hr., which increases the value found with *L. arabinosus,* but leaves that obtained with *L. casei* unchanged (164). It is probable that the marked variations in the biotin content of yeast extract, as determined with various test organisms in a recent study (165), reflected variable utilization by the various organisms of incompletely hydrolyzed combinations of biotin, since such variation is not apparent when more rigorous hydrolytic treatment is employed (164).

From results cited above, procedures of choice for extraction of biotin would appear to be (a) hydrolysis at 120° C. (15 lb. pressure in the autoclave) for 2 hr. with 2-4 N H_2SO_4 (163), or (b) hydrolysis at 120° C. for 1 hr. with 6 N H_2SO_4 (164). In some cases, even this treatment may not be sufficiently drastic (166). Alkaline hydrolysis gradually destroys biotin (166).

Oleic acid, and other lipoidal materials which contain this fatty acid, markedly interfere with biotin determinations with *L. casei* (167, 168), and with all other lactic acid bacteria so far tested (168a). Linoleic and linolenic acids are also effective. Under appropriate conditions (168a), biotin is no longer required for growth of these organisms when these fatty acids are present in the medium. It is thus essential that fatty materials be removed from samples for assay. This is easily accomplished by filtration of the hydrolyzed extract at pH 4.5 through paper, as described under riboflavin and pantothenic acid assay. This procedure has proved effective also for biotin (167).

Except for the action of lipoidal materials, cited above, the response of lactic acid bacteria to biotin appears quite specific. No related structure of proved natural occurrence can replace the vitamin for these organisms. Desthiobiotin, biotin sulfoxide, biotin sulfone, and the diaminocarboxylic acid derived from biotin by opening the ureido ring, all are ineffective in promoting growth (169, 170, 164). In high concentration, certain of these derivatives may inhibit the response of some lactic acid bacteria to limited amounts of biotin (171), but such concentrations can scarcely occur in natural products. A synthetic analogue of biotin, oxybiotin, which has high growth-promoting activity for some lactic acid bacteria, apparently does not occur naturally (172, 173). A number of these products have some activity in promoting growth of yeast, but no evidence is available to show that their concentration in natural materials is sufficiently high to interfere seriously with the use of yeast growth methods for determining biotin. Preliminary evidence for the widespread occurrence in natural products of avidin-uncombinable "vitamers" of biotin, active in promoting growth of yeast and some other microorganisms (174), could not be confirmed (175), and has never been presented in detail. Such a substance does occur in urine, however (175). The subject needs further investigation.

(2) Assay Media for Biotin and Their Development

Because of the widespread occurrence of biotin and its chemical stability, purified or semisynthetic media were necessary before a direct demonstration of its essential nature could be made. This vitamin was first found to be among those required for lactic acid bacteria by Möller (176), who showed in 1939 that *Streptobacterium plantarum (Lactobacillus plantarum)* required it for growth. As the nutritive requirements of lactic acid bacteria became better known, it was found that each of these organisms requires an external source of this vitamin (49, 48, 33). Since discovery of the biotin-binding protein, avidin (177), it has been possible to demonstrate the biotin requirement of numerous organisms in crude natural media (178). The possibility of using lactic acid bacteria for determination of the vitamin was first pointed out by Snell and Wright (48), who showed that, by adding nicotinic acid and omitting biotin from their assay medium for nicotinic acid (Medium 8, Table IV), *Composition of Media for Assay of Biotin*

Constituent	23. Landy and Dicken, 1942 (49) For *L. casei*	24. Shull, Hutchings and Peterson, 1942-3 (179, 180) For *L. casei*	25. **Wright and Skeggs, 1944** (164) For *L. arabinosus*	26. Luckey, Moore and Elvehjem, 1946 (181) For *L. arabinosus* and *S. faecalis*
	Amount/100 cc. of double strength medium			
Casein, acid-hydrolyzed, charcoal-treated	1.0 g.	—	1.0 g.	1.0 g.
Casein, acid-hydrolyzed,[a] peroxide-treated	—	1.0 g.	—	—
Yeast extract, charcoal-treated,[a] peroxide-treated	—	0.30 g.	—	—
Glucose	2.0 g.	2.0 g.	4.0 g.	2.0 g.
Sodium acetate (anhydrous)	1.2 g.	2.0 g.	1.2 g.	0.8 g.
Asparagine	50 mg.	40 mg.	—	—
Cystine	20 mg.	20 mg.	20 mg.	20 mg.
Tryptophan	20 mg.	15 mg.	20 mg.	40 mg.
Adenine	1.0 mg.	2.0 mg.	1.0 mg.	2.0 mg.
Guanine	1.0 mg.	2.0 mg.	1.0 mg.	2.0 mg.
Xanthine	1.0 mg.	—	1.0 mg.	2.0 mg.
Uracil	1.0 mg.	—	1.0 mg.	2.0 mg.
p-Aminobenzoic acid	—	20 γ	20 γ	20 γ
Calcium pantothenate	40 γ	40 γ	200 γ	100 γ
Folic acid	0.3 γ [c]	—	—	0.4 γ
Nicotinic acid	40 γ	200 γ	200 γ	160 γ
α-Pyracin	—	—	—	0.4 γ
Pyridoxine hydrochloride	80 γ	40 γ	400 γ	100 γ
Pyridoxal hydrochloride	—	—	—	0.4 γ
Riboflavin	40 γ	20 γ	200 γ	100 γ
Thiamine chloride	20 γ	—	200 γ	100 γ
Inorganic salts				
Solution A [b]	1.0 cc.	1.0 cc.	1.0 cc.	—
Solution B [b]	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
K_2HPO_4	—	—	—	1.0 g.

[a] See text for preparation. [b] As in Medium 8, Table IV. [c] Recalculated in terms of pure folic acid.

biotin could be determined with *L. arabinosus.* Subsequently, various media for determination of this vitamin with *L. casei* (49, 179, 180) were devised, and a modified medium for use with *L. arabinosus* also presented (164). The composition of these latter media is shown in Table IX. It should again be noted that media suitable for *L. casei* will also support growth of *L. arabinosus;* the converse, however, is not necessarily true.

(3) Condensed Summary of Assay Methods for Biotin Employing Lactic Acid Bacteria

a. Method of Landy and Dicken (49). Medium 23, Table IX, is used with *Lactobacillus casei.* Since the method was presented as a general procedure for assay of several vitamins, and has not been especially studied from the standpoint of biotin determination, it will be described in a later section under *generalized methods* (*cf.* p. 418).

b. Method of Shull, Hutchings and Peterson (179), as Modified by Shull and Peterson (180).

Assay organism—*Lactobacillus casei,* ATCC No. 7469. Carried as stab cultures by monthly transfer in Medium "b," Table II.

Basal medium—Medium 24, Table IX. Initial pH of medium, 5.6.

Preparation of unusual ingredients of medium—

(1) Peroxide-treated casein hydrolyzate—100 g. of Labco vitamin-free casein, 375 cc. of water, and 125 cc. concentrated H_2SO_4 are mixed uniformly and autoclaved at 15 lb. pressure for 15 hr. 640 g. of $Ba(OH)_2.8H_2O$, suspended in 400 cc. of boiling water, are added to the hydrolyzate and the precipitate of $BaSO_4$ filtered out with Filter-Cel. The filter cake is triturated with 500 cc. of hot water, filtered, and these washings added to the main filtrate. The pH is adjusted to 3.0 with NaOH, the solution diluted to 1500 cc., and 15 cc. of superoxol (30% H_2O_2) are added. The solution is stirred, then allowed to stand at room temperature for 24 hr. After adjusting with NaOH to pH 7.0, 1% of powdered MnO_2 is added. The solution is stirred mechanically until oxygen evolution ceases (*ca.* 15 min.), filtered with suction, and diluted to 2000 cc., 1 cc. ≎ 50 mg. casein.

(2) Peroxide-treated yeast filtrate—To 20 g. of Difco yeast extract dissolved in water and adjusted to pH 2.0 with H_2SO_4 are added 4.0 g. of activated charcoal (Norit A). The mixture is stirred mechanically at 55-60° C. for 30 min. The Norit is filtered out and the filtrate (A) saved for later use. The Norit is stirred for 15 min. at room temperature with 200 cc. of 50% ethanol, the charcoal filtered out, and the filtrate (which contains unwanted biotin) discarded. The Norit is eluted a

second time by stirring 15 min. at 55-60° C. with 200 cc. of NH_4OH (28%) alcohol-water mixture (10:50:40 by volume) and filtering. The Norit is discarded. The eluate is distilled *in vacuo* to remove ethanol and NH_3, then diluted to 200 cc. with water and adjusted to pH 3.0. 10 g. of Super Filtrol (Filtrol Corporation, Los Angeles) are added; the mixture is stirred at room temperature for 15 min., then filtered. The filtrate is discarded, and the Super Filtrol eluted by stirring with 200 cc. of the ammoniacal ethanol solution for 15 min. at room temperature. After filtering, the Super Filtrol is discarded, the eluate concentrated *in vacuo* to remove NH_3 and ethanol, then added to the original filtrate (A) above. The solution is diluted to 1300 cc., adjusted to pH 3.0, and 13 cc. of superoxol added. After standing for 24 hr. at room temperature, the pH is adjusted to 7.0 and 15 g. of MnO_2 are added with stirring. When the evolution of oxygen ceases, the solution is filtered and is ready for use. This supplement provides the chief source of Norit eluate factor (folic acid, *L. casei* factor) for the assay medium. Its adequacy can be tested by supplementing the basal medium (supplemented with 0.001 γ biotin/10 cc.) with 0.5-3.0 cc. of the solution. These amounts should permit production of about 11.0 cc. of $N/10$ acid after a 3 day incubation period. The supplement should be used in amounts somewhat larger than necessary to permit production of this amount of acid in assay tubes containing excess biotin.

Inoculum medium—Contains Difco yeast extract 0.5%, sodium acetate 0.6%, glucose 0.5% and 0.05 cc. each of Salts A and B/10 cc. (as in medium 8, Table IV). Tubed in 10 cc. lots and sterilized as usual.

Inoculum—Incubated 20-30 hr. at 37° C. Cells are then centrifuged out, resuspended in 10 cc. of sterile water, and one drop (0.05 cc.) of this suspension added to 10 cc. of sterile water. One drop of this latter dilute suspension is used to inoculate each assay tube.

Concentration of biotin for standard curve—0, .04, .07, .10, .15, .20, .25, .40, .70, 1.00 mγ biotin/10 cc. of diluted medium. Samples should be run at several levels falling between the extremes of concentration.

Sterilization—Autoclaved 15 min. at 15 lb. pressure.

Incubation time and temperature—3 days at 37° C.

Measurement of response—Titration with 0.1 N NaOH.

Comment—Peroxide treatment of casein and yeast supplement is carried out to destroy the remaining biotin by oxidation. The yeast supplement is necessary to supply folic acid (*L. casei* factor), which is required for growth of *L. casei*, and which was not readily available when this method was devised. The tedious adsorptions, elutions, *etc.*, are necessary to obtain a source of this substance uncontaminated with biotin. Use of synthetic folic acid is now possible, and would permit elimination of the yeast supplement from the medium. However, growth with pure folic acid is not quite as good as with the yeast supplement (182), indicating that the latter supplies stimulative factors in addition to folic acid. Others have also noted the response of *L. casei* on somewhat similar media to stimulative factors (183, 184). In the author's opinion, *L. arabinosus* is a much superior organism for the determination of biotin, since it does not require folic acid and is less affected by stimulative factors of unknown nature than is *L. casei.* In practice, however, both organisms give closely similar values for biotin when used on properly prepared extracts (164).

c. *Method of Wright and Skeggs (164).*

Assay organism—*Lactobacillus arabinosus,* ATCC 8014. Carried as stab cultures by monthly transfers in Medium "b," Table II.

Basal medium—Medium 25, Table IX. Initial pH not specified, but approximately 6.8.

Preparation of unusual ingredients of medium—

(1) Hydrolyzed casein, charcoal-treated—Prepared as described under "Method for Nicotinic Acid," p. 364.

Inoculum medium—1% glucose, 1% yeast extract (Difco). Tubed in 10 cc. lots and sterilized as usual.

Inoculum—Incubated at 30° C. for 18-36 hr. before use. Cells are centrifuged out, resuspended in sterile saline, and again centrifuged out. A faintly visible suspension of these washed cells in sterile saline is prepared. One drop of this is used to inoculate each assay tube.

Concentration of biotin for standard curve—0, .25, .50, 1.0, 1.5, 2.0 mγ of biotin/10 cc. of medium. Samples are run at 4 or more concentration levels calculated to fall within this range.

Sterilization—Autoclaved at 15 lb. for 15 min.

Incubation time and temperature—Approximately 72 hr. at 30-33° C.

Measurement of response—Titration of lactic acid produced with standard alkali.

Comment. Of the procedures presently available, this yields perhaps the most uniformly successful results. The same organism can also be used for assay in media suitable for *L. casei,* but little advantage is apparent in using the more complex media. The method has been critically discussed elsewhere (183a).

d. *Method of Luckey, Moore and Elvehjem (181).*

Assay organism—*Lactobacillus arabinosus,* ATCC No. 8014 or *Streptococcus faecalis* R, ATCC No. 8043.

Basal medium—Medium 26, Table IX. Initial pH of medium 6.8.

Preparation of unusual ingredients of medium—
(1) Acid hydrolyzed casein—Prepared as described under "Method for Nicotinic Acid," p. 364, with the modification that NH_4OH is used in place of NaOH for all neutralizations.

Inoculum medium—Not specified, but apparently 5 cc. of basal medium, 0.004 γ biotin and water to 10 cc. for each inoculum tube.

Inoculum—Incubated 24 hr. at 30° C., cells centrifuged out, resuspended in sterile saline (equal volume?) and one drop of the resulting suspension used to inoculate each assay tube.

Concentration of biotin for standard curve—0-0.004 γ biotin/10 cc. of medium for *L. arabinosus,* 0-0.0015 γ biotin for *S. faecalis.*

Sterilization—Autoclaved at 15 lb. pressure for 15 min.

Incubation time and temperature—16-20 hr. at 30° C.

Measurement of response—Turbidimetric measurement of growth, *e.g.*, with the Evelyn colorimeter.

Comment—The medium used in this assay is very similar to that used for *L. arabinosus* by Wright and Skeggs (Medium 24). The only important modification is the addition of folic acid, which is required for growth of *S. faecalis.* Addition of pyridoxal is superfluous where pyridoxine is used at the recommended level, while α-pyracin has no effect on growth and need not be added.

The assay was originally presented as a means for differentiating between natural biotin and the synthetic analogue, oxybiotin. For *L. arabinosus, dl*-biotin and *dl*-oxybiotin are almost equally active, while for *S. faecalis,* oxybiotin is essentially inactive ($< 1/20$ as active as biotin). With one or two unexplained variations, the biotin content of natural materials was identical as determined with both organisms; no evidence for natural occurrence of oxybiotin could be secured (181).

It is probable that the amount of growth produced in response to biotin by *S. faecalis* could be greatly increased by substituting 2% sodium citrate for the 0.4% of sodium acetate present in this medium (*cf.* 67). Whether this would increase the precision of the assay cannot, of course, be stated at present.

(4) Other Lactic Acid Bacteria Available for Assay of Biotin

All lactic acid bacteria whose requirements have been investigated require biotin for growth (38, 42, 33, 22, 178). Of the many organisms thus available, only *L. casei, L. arabinosus,* and *S. faecalis* have been investigated as assay organisms, and each has proved useful in this capacity. It is evident that many additional organisms might also be used in this fashion.

F. FOLIC ACID (VITAMIN B_c, L. CASEI FACTOR, VITAMIN M, PTEROYLGLUTAMIC ACID, ETC.)

(1) *Extraction from Natural Materials*

The existence in natural materials of a substance having the biological properties now associated with the pure compound, pteroylglutamic acid, was discovered independently by a number of different workers, using as test organisms monkeys (185), chicks (186, 187) and the lactic acid bacteria, *Lactobacillus casei* (14a) and *Streptococcus faecalis* (15). Concentration and eventual isolation of several related compounds having activity was achieved by using these two microorganisms as test objects. A summary of the forms of this vitamin known to occur naturally, and of their activity for *L. casei* and *S. faecalis,* is given in Table X. The parent vitamin is the structure, pteroylglutamic acid, and has high activity for all test organisms. As the number of glutamic acid residues in the chain increases, the activity falls to low levels for both *L. casei* and *S. faecalis;* the fall is much more rapid for the latter organism, as evidenced by the comparative activities of pteroyldiglutamylglutamic acid for the two organisms. The SLR factor (rhizopterin) is inactive for animals; it is, however, converted to a substance (apparently pteroylglutamic acid) which has activity for *L. casei* by organisms which utilize it for growth (189, 190). In contrast to these microorganisms, chicks (191), monkeys (192, 193, 194), and presumably some other animals, can utilize the conjugated forms of folic acid as readily as the parent compound. However, the fox provides an example of an animal which cannot utilize the conjugated forms of folic acid in yeast (195) or the crystalline pteroylheptaglutamate. Conflicting reports of the activity of folic acid conjugates in pernicious anemia have been published (196), (201), but recent evidence indicates that they can be used. These differences in availability of the known conjugated forms of folic acid, and the probable existence of unidentified conjugated forms of unknown availability to animals, obviously complicates greatly the problem of obtaining microbiological assays for folic acid which are meaningful in terms of animal nutrition. Since known and unknown conjugates make up a very considerable portion of the total vitamin activity for most animals, however (197, 198, 194), the tendency has been to prepare digests for assay in which as much as possible of the bound folic acid has been converted to the free form, and thus to obtain reliable values for the total folic acid present. How well present procedures accomplish this remains to be seen.

Folic acid is destroyed by acid and alkali (15); these reagents cannot, therefore, be used for its release from tissues. Digestion with takadiastase or a mixture of takadiastase and papain releases additional microbiologically available folic acid from a large number of materials (78, 196). Autoclaving an extract at an initial pH of 4.0 for 12 hr. at

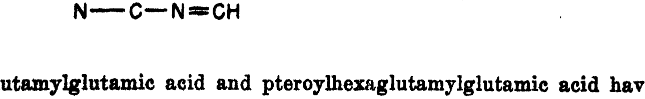
TABLE X

Growth-Promoting Activity of Various Naturally Occurring Forms of Folic Acid for L. casei and S. faecalis

Compound	Chemical nature	Approximate relative activity for[a]		References
		L. casei.	*S. faecalis*	
Liver *L. casei* factor Vitamin B_c Folic acid	Pteroylglutamic acid[b]	100	100	(15, 18, 199, 200, 201)
Fermentation *L. casei* factor	Pteroyldiglutamylglutamic acid	85	6	(202, 200, 201)
Vitamin B_c conjugate	Pteroylhexaglutamylglutamic acid	0.3-0.8	0.2	(191, 101, 102)
SLR factor (rhizopterin)	Formylpteroic acid[c]	$<$0.01	100	(16, 189, 190)

[a] Relative activity of 100 for *L. casei:* compound gives approximately ½ maximum growth in a concentration of 0.5 mγ/10 cc. of medium under conditions of testing. Relative activity of 100 for *S. faecalis:* compound gives approximately ½ maximum growth in a concentration of 0.5-2.5 mγ/10 cc. of medium under test conditions. The amount required to promote half maximum growth varies considerably under different assay conditions; the figures are illustrative of magnitudes only, and are not to be considered exact.

[b] Pteroylglutamic acid has the formula:



Pteroyldiglutamylglutamic acid and pteroylhexaglutamylglutamic acid have 3 and 7 glutamic acid residues in place of the one present in pteroylglutamic acid.

[c] Pteroic acid (Formula I above, but with no glutamic acid in molecule) has very similar activity relationships, in that it is active for *S. faecalis,* inactive for *L. casei* (200). The formyl group of the SLR factor (formylpteroic acid) replaces the hydrogen atom on the nitrogen of the p-aminobenzoic acid residue of pteroic acid.

15 lb. pressure is effective in releasing folic acid from certain liver preparations (203).

Most generally applicable, and therefore of greatest value, is digestion with concentrates of certain naturally-occurring enzymes. The normal substrates for these enzymes appear to be conjugates of folic acid, which are hydrolyzed to the parent compound, pteroylglutamic acid. Such enzymes are distributed fairly widely and fall into two classes: (1) the type present in chicken pancreas, having a pH optimum at 7.0-8.0 (204, 198) and (2) the type present in most other animal tissues, especially rich in liver and kidney, with an optimum pH around 4.5 (197, 204, 198). Both types have been used to prepare samples for microbiological assay, but in view of the source materials required, type 2 is the most practicable for use. Certain materials (*e.g.*, yeast extract) contain inhibitors for the conjugate-splitting enzyme; to release maximal amounts of free folic acid from such materials, comparatively large amounts of the enzyme are required (204).

Because of these and other complexities, which have not been thoroughly studied, it is not possible to give a single extraction procedure which will give maximum assay values with all samples. The procedure given below is perhaps the most useful and practicable from the standpoint of most laboratories; values obtained with it checked well those obtained by chick assay of the same materials (197). An alternative procedure is given by Mims, Totter and Day (205).

Recent evidence indicates, however, that in many materials these procedures, too, fail to yield maximum assay values for folic acid. Generally applicable procedures which will do so remain to be developed.

a. Extraction Method of Bird et al. (197). The conjugate-splitting enzyme from hog kidney is used. To prepare the enzyme, fresh hog kidney is ground and blended (*e.g.*, in a Waring Blendor) with 3 parts of distilled water. The resulting suspension is centrifuged, and the supernatant layer poured off and filtered through Super-Cel. This clarified extract may be dried from the frozen state and stored in the refrigerator, or, more simply, dispensed in 5 cc. lots in test-tubes and maintained in the frozen state in a dry ice chest until use. It appears to be stable under these conditions for at least several months.

The finely ground sample is diluted with water so that an amount estimated to contain 1-2 γ of the vitamin is present in 1 or 2 cc. of water. To this sample is added 5 cc. of 0.1 *M* acetate buffer of pH 4.5.[32] An amount of enzyme preparation equivalent to approximately 0.4 g. of fresh hog kidney is now added, followed by a few drops of toluene. The flask or test tube is stoppered and incubated 16 hr. at 45° C. The contents are then adjusted to pH 7.0 with *N* NaOH, the volume adjusted to 10 cc. with water, and the whole autoclaved at 15 lb. for 5 min. Appropriate dilutions are then made for assay. Blanks containing only the enzyme preparation should be run at intervals; if its folic acid content is significant, this must be subtracted from that found for the sample.

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[32] With many samples, especially those containing unbroken cells, such as yeasts or other microorganisms, it is well to autoclave at 15 lb. pressure for 15 min. at this point, to extract cellular constituents and render them available to action of the enzyme, which is added after cooling (unpublished data).

b. Checking for Complete Hydrolysis. The fact that conjugates of folic acid of comparatively low molecular weight, such as pteroyldiglutamylglutamic acid are relatively much more active for *L. casei* than for *S. faecalis* has been mentioned above. Data of Bird and Robbins (101) show also that vitamin B_c conjugate (and presumably other conjugates of folic acid) are relatively more active for *L. casei* at high dosage levels than at low dosage levels. When samples containing such conjugates are assayed with *L. casei* against a standard of the free vitamins, pronounced drifts are apparent. If all of the vitamin in a sample is present in the free form, therefore, values obtained with *L. casei* should be relatively constant at increasing levels of the vitamin, *i.e.*, "drift" should be at a minimum.[33] Furthermore, unless all of the vitamin is present in the free form, values obtained with *S. faecalis* will be lower than those obtained with *L. casei*.[34] Thus, the following cases may occur: (a) the *L. casei* assay is relatively free of drift, and checks the *S. faecalis* assay all of the vitamin *in the extract* is present in the free form; little or no SLR factor (rhizopterin) (16) is present; (b) the *L. casei* assay shows considerable drift, and gives higher values than assay with *S. faecalis:* conjugated forms of folic acid are present; the *L. casei* value most nearly represents total vitamin activity, the *S. faecalis* assay approximates most closely the content of free pteroylglutamic acid; (c) assay with *L. casei* gives a lower result than assay with *S. faecalis:* substances such as the SLR factor are present, which are active for *S. faecalis* but not for *L. casei*. Intermediate or mixed cases may also occur.

The above generalities may perhaps hold true in the simpler cases. However, we probably do not know all of the forms in which this vitamin occurs naturally, and further work will be necessary to establish that complete extraction of folic acid has been achieved in any case, and to explain the reasons for certain discrepancies which occur in the assay values obtained with the two test organisms.

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[33] "Drift" may occur for reasons other than the presence of conjugated forms of folic acid in the sample, however. See p. 350-52.

[34] This is strictly true only in samples which contain negligible amounts of SLR factor, which is active for *S. faecalis* but not for *L. casei* (16). Little has been published concerning distribution of this substance.

The microbiological assays for folic acid are not completely specific. Thymine or thymidine in adequate amounts can completely replace folic acid for *S. faecalis* (15, 206, 207, 208), and for *L. casei* (104, 208, 209) in assay media which contain purine bases. In samples containing significant quantities of thymine, high assay values for folic acid may be obtained (104).

(2) Assay Media for Folic Acid and Their Development

The medium used by Snell and Peterson (14a) in the initial work on concentration of the "Norit eluate factor" (folic acid) was developed at a time when certain of the growth factors required for this organism were unknown. These were supplied as crude materials, freed from folic acid by charcoal adsorption. Though satisfactory for assay of concentrates of folic acid, the medium was recognized as marginal in certain constitutents, since it did not support heavy growth of *L. casei*, as did, for example the riboflavin assay medium when supplemented with riboflavin. It is not suitable for assay of crude substances for their folic acid content. Subsequently, more complete and very similar media for the assay of folic acid with *S. faecalis* or *L. casei* were developed by Mitchell and Snell (207) and Landy and Dicken (49), respectively. The latter authors showed that the chief deficiencies in the earlier medium of Snell and Peterson for *L. casei* were asparagine and biotin, the last of which had become available since the earlier work. Subsequent experience with media for these two organisms has resulted in further slight modifications which increase somewhat the rate and extent of growth obtained. The composition of the Landy-Dicken medium has been given (Medium 23, Table IX). For folic acid assay, the folic acid is omitted and biotin is added at a level of 1.0 γ for 100 cc. of double strength medium. The composition of other media for assay of folic acid is given in Table XI.

At present, no assay method or medium for folic acid has been exhaustively or critically tested for the accuracy of assays obtained with it. This is principally because, with very few exceptions (197), no figures for the folic acid content of natural materials obtained by independent methods are available. For lack of better criteria, it has been assumed that the media giving the best growth of the test organisms and the highest assay values for folic acid are most suited for use. By these standards, Media 29 and 30 are superior to those devised earlier (cf. also 209a).

(3) Condensed Summary of Assay Methods for Folic Acid Employing Lactic Acid Bacteria

a. Method of Mitchell and Snell (207).

Assay organism—*Streptococcus faecalis* R, ATCC No. 8043. Carried as stab cultures by monthly transfer in Medium "b," Table II.

Basal medium—Medium 27, Table XI. Initial pH 6.8.

Preparation of hydrolyzed casein for basal medium—Prepared by HCl hydrolysis as described under "Method for nicotinic acid," p. 364.

Inoculum medium—5 cc. of basal medium, 1.0 mg. of liver extract (Wilson and Co., Fraction "B")[35] and 5 cc. of water per tube. Sterilized by autoclaving as usual and held until use.

Inoculum—Incubated at 30° C. for 14-30 hr. before use. Cells are centrifuged out, the supernatant medium poured off, and cells resuspended in approximately 20 cc. of sterile saline. One drop of this suspension is used to inoculate each assay tube.

Concentration of folic acid for standard curve—In the original fractionation, an arbitrary standard (liver Fraction B) was used at levels of 0, 0, 20, 40, 60, 100 and 200 γ/10 cc. of final medium. In terms of pure folic acid this is approximately equivalent to 0, 0, 0.15, 0.30, 0.50, 0.75, 1.0 and 1.5 mγ of pure pteroylglutamic acid.

Sterilization—Autoclaved at 15 lb. pressure for 15 min.

Incubation time and temperature—16 hr. at 30° C.

Measurement of response—Turbidimetric.

Comment—The procedure served admirably as a test in following the concentration of folic acid (15). The maximum growth obtained is, however, not great. To obtain best growth, the recommended autoclaving time must be observed, since, with shorter times, considerably more vitamin B_6 must be present in the medium (210). Growth of this organism is greatly favored by use of citrate buffer in place of acetate, as in Medium 29. For routine assay of crude materials, the later modified medium will be found more satisfactory than this early medium.

Bird *et al.* (197) have used a slight modification of this method for assay of folic acid with *L. casei*. In this procedure the pyridoxine is raised from 20 to 100 γ/100 cc., the inoculum is grown in the riboflavin medium (Medium 2, Table IIIa) of Snell and Strong, supplemented with 1 γ of riboflavin/10 cc. of final medium, and response to folic acid is determined acidimetrically after 3 days' growth.

b. Method of Landy and Dicken. Medium 23, Table IX, is used with

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[35] Added as a source of folic acid: small amounts of the pure vitamin or of other crude materials could be used with equal success.

Lactobacillus casei. The method was presented as a general procedure for several vitamins and will be considered in the section on *generalized methods* (p. 418).

c. Method of Luckey, Briggs, and Elvehjem (211).

Assay organism—*Streptococcus faecalis* R, ATCC 8043. Method of carrying stock culture not specified; apparently as described by Mitchell and Snell (procedure a above).

Basal medium—Medium 28, Table XI. Initial pH 7.0-7.2 (KOH used for the final adjustment).

Preparation of hydrolyzed casein for assay medium—Prepared by HCl hydrolysis as described under "Method for nicotinic acid," p. 364.

Inoculum medium—5 cc. of basal medium 200 γ of solubilized liver (Wilson's fraction "B" or "L") and 5 cc. of water per tube; sterilized 15 min. at 15 lb. pressure.

Inoculum—Incubated at 30° C. for 8-30 hr. Suspended uniformly and 1 drop of the suspension used directly to inoculate each assay tube.

Concentration of folic acid for standard curve—0-300 γ of solubilized liver in graded doses (equivalent to approximately 0-2.0 mγ of pure pteroylglutamic acid/10 cc. of final medium.)

Sterilization—15 lb. for 15 min.

Incubation time and temperature—16 hr. at 30° C.

Measurement of response—Turbidimetric.

Comment—The medium permits somewhat better growth than the original medium of Mitchell and Snell (207) but is inferior in this respect to the subsequent modification by Teply and Elvehjem (67).

d. Method of Teply and Elvehjem (67).

Assay organism—*Streptococcus faecalis* R ATCC 8043 or *Lactobacillus casei,* ATCC No. 7469. Method of carrying cultures not specified.

Basal medium—For *S. faecalis:* Medium 29a, Table XI. For *L. casei:* Medium 29b, Table XI. Except for the buffers, the latter medium may be used for both organisms. However, the asparagine, alanine, *p*-aminobenzoic acid, peptone, and salts B are not necessary or helpful for *S. faecalis* and may be omitted for this organism as indicated in Medium 29a, if desired.

Preparation of special ingredients of the assay medium—

(1) Hydrolyzed casein—Prepared by hydrolysis with HCl, as described under "Method for nicotinic acid," p. 364.

(2) Charcoal-treated peptone—50 g. of peptone in 200 cc. of water are adjusted to pH 3.0 and stirred with 5 g. of Norit for 1 hr., filtered, and diluted to 500 cc. The solution may be preserved under toluene. This

TABLE XI

Composition of Media for Assay of Folic Acid

Constituent	Medium No. 27. Mitchell and Snell, 1941 (207) (for *S. faecalis*)[a]	28. Luckey, Briggs and Elvehjem, 1946 (211) (for *S. faecalis*)	29. Teply and Elvehjem, 1945 (67) (a) (for *S. faecalis*)	29. Teply and Elvehjem, 1945 (67) (b) (for *L. casei*)	30. Robert and Snell 1946 (51) (for *L. casei*)[b]
	Amount/100 cc. of double strength medium				
Casein, acid-hydrolized, charcoal-treated	1.0 g.	1.0 g.	1.0 g.	1.0 g.	—
Casein, pancreatin-digested, charcoal-treated	—	—	—	—	4.0 g.
Peptone, charcoal-treated	—	—	—	20-60 mg.[c]	—
Glucose	2.0 g.	2.0 g.	4.0 g.	4.0 g.	4.0 g.
Sodium acetate (anhyd.)	1.2 g.	0.4 g.	—	4.0 g.	4.0 g.[d]
Sodium citrate	—	—	5.0 g.	—	—
DL-Alanine	—	—	40 mg.	40 mg.	—
Asparagine	—	—	20 mg.	20 mg.	—
Cystine	20 mg.	20 mg.	40 mg.	40 mg.	20 mg.
Tryptophan	10 mg.	60 mg.	40 mg.	40 mg.	—
Adenine	2 mg.	2 mg.	2 mg.	2 mg.	2 mg.
Guanine	2 mg.	2 mg.	2 mg.	2 mg.	2 mg.
Xanthine	2 mg.	2 mg.	2 mg.	2 mg.	—
Uracil	2 mg.	—	2 mg.	2 mg.	2 mg.
p-Aminobenzoic acid	—	—	2 γ	2 γ	20 γ
Calcium pantothenate	20 γ	80 γ	80 γ	80 γ	100 γ
Nicotinic acid	20 γ	120 γ	120 γ	120 γ	100 γ
Pyridoxine hydrochloride	20 γ[a]	240 γ	240 γ	240 γ	200 γ
Riboflavin	40 γ	40 γ	40 γ	40 γ	100 γ
Thiamine chloride	20 γ	40 γ	40 γ	40 γ	100 γ

TABLE XI (continued)

Constitutent	Medium No.				
	27. Mitchell and Snell, 1941 (207) (for *S. faecalis*) [a]	28. Luckey, Briggs and Elvehjem, 1946 (211) (for *S. faecalis*)	29. Teply and Elvehjem, 1945 (67) (a) (for *S. faecalis*)	29. Teply and Elvehjem, 1945 (67) (b) (for *L. casei*)	30. Roberts and Snell 1946 (51) (for *L. casei*) [b]
	Amount/100 cc. of double strength medium				
Biotin	0.04 γ	0.08 γ	0.08 γ	0.08 γ	0.8 γ
Inorganic salts					
Solution B[e]	1.0 cc.	—	1.0 cc.	1.0 cc.	—
Solution C	—	—	—	—	4.0 cc.[f]
K_2HPO_4	0.05 g.	1.0 g.	0.5 g.	0.5 g.	0.5 g.
KH_2PO_4	0.05 g.	—	—	—	0.5 g.

[a] From subsequent developments in the field of vitamin B_6 (12, 13, 210), the amount of pyridoxine present in this medium is known to be inadequate if short autoclaving periods are used, or if *L. casei* is used as the test organism. Bird and coworkers (197) have used this medium for assay with *L. casei* by raising the pyridoxine concentration from 20 to 100 γ/100 cc. (see text) *cf.* also Media 28-30.

[b] Presented as a general assay medium; omission of any of the essential vitamins permits assay of that particular vitamin. When used for vitamins other than folic acid, 0.4 γ of pure folic acid (pteroylglutamic acid) should be added for each 100 cc. of double strength medium.

[c] The amount used is variable; see text.

[d] When the tryptic casein digest is prepared as described in the text, it contains the proper amount of sodium acetate; no further additions should be made.

[e] As in Medium 8, Table IV.

[f] The solution of salts C has the following composition: $MgSO_4.7H_2O$, 10 g.; NaCl, 0.5 g.; $FeSO_4.7H_2O$, 0.5 g.; $MnSO_4.4H_2O$, 2.0 g.; water to make 250 cc. It differs from Salts B of previous media in containing a higher concentration of Mn^{++}.

preparation is added to the medium at the maximum level possible without producing an excessively high blank titration (> 3.0 cc.) This amount usually lies between 1 and 3 mg. per tube.

Inoculum medium—For *S. faecalis:* 5 cc. of basal medium, 1.0 mg. of solubilized liver and 5 cc. of water per tube. For *L. casei:* 5 cc. of basal medium, 0.1 mg. solubilized liver, and 5 cc. of water per tube. Sterilized as usual and stored until use.

Inoculum—*S. faecalis:* Incubated 8-30 hr. at 30° C. *L. casei:* incubated approximately 24 hr. at 37° C. (Latter point not specified.) One drop of the proper suspension used directly to inoculate each assay tube.

Concentration of folic acid for standard curve—For *S. faecalis:* 0, 0.5, 1, 2, 3, 4, 5, 6, and 8 mγ vitamin B_c (pteroylglutamic acid)/10 cc. of final medium. For *L. casei:* 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mγ vitamin B_c/10 cc. of final medium.

Sterilization—Autoclaved at 15 lb. for 15 min.

Incubation time and temperature—For *S. faecalis:* 30-72 hr. at 30° C. For *L. casei:* 30-72 hr. at 37° C.

Measurement of response—Titration with 0.1 *N* alkali.

Comment—The citrate buffered medium for *S. faecalis* permits much higher acid production, as well as increased growth, by this organism than do previous media. Citrate cannot be substituted for acetate in medium 29b for *L. casei*, since no growth is obtained under these conditions.

The charcoal-treated peptone preparation supplies stimulatory substances ("strepogenin," *etc.*) which greatly increase regularity, speed, and extent of growth of *L. casei.*

e. Method of Roberts and Snell (51).

Assay organism—*Lactobacillus casei* ATCC No. 7469. Carried as stab cultures with biweekly transfer in medium "d," Table II.

Basal medium—Medium 30, Table XI. Initial pH of medium, 6.8-7.0.

Preparation of trypsin-digested casein for basal medium—120 g. of purified casein (Labco, "vitamin-free") are dissolved by *gradual* addition, with shaking, to 2 l. of 0.8% $NaHCO_3$ solution. The pH of the suspension should be measured; if below 8.0, it should be brought to the latter value by addition, with stirring of NaOH solution. To the uniform suspension thus obtained, 600 mg. of pancreatin suspended in 15-20 cc. of distilled water are added. The mixture is covered with a thin layer of toluene, shaken well, and allowed to stand for 48 hr. at 37° C. It is then placed in the autoclave and heated in flowing steam for 20-30 min. to remove the toluene. After cooling to room temperature, the solution is adjusted to pH 6.0 by addition of glacial acetic acid (about 7.0 cc.), and filtered with mild suction. To the filtrate, 60 g. of activated charcoal (*e.g.*, Darco G-60) are added. The mixture is stirred for 30 min., then filtered with suction. The filtrate often contains colloidal charcoal which is removed during the next step. Glacial acetic acid (about 75 cc.) is now added to adjust the pH to 3.0-3.8, 24 g. of activated charcoal are added, the mixture is stirred for 30 min., then filtered. Filtrations are aided by use of Hyflo Super Cel. The residue from each filtration is washed with 50-75 cc. of water; washings are added to the main filtrate. The final clear filtrate is diluted to 2.4 l. (1 cc. ≡ 50 mg. casein) and preserved under toluene. Dry weight determinations should be made on small aliquots of each lot of casein digest prepared in this way. Unless 40-50 mg. of solids are present in each cc. of solution, the preparation is unsatisfactory and should be repeated.

Inoculum medium—5 cc. of basal medium, 0.02 γ folic acid, and 5 cc. of water per tube. Plugged with cotton, sterilized at 15 lb. pressure for 10 min., and stored until use.

Inoculum—Incubated 16-24 hr. at 37° C. Cells are thrown down by centrifugation, the supernatant liquid removed, and the cells resuspended in 10 cc. of sterile 0.9% NaCl solution. A sufficient amount (usually about 0.1-0.3 cc.) of this heavy suspension is added to a second 10 cc. portion of sterile saline to give a suspension which is just visibly turbid. One drop of this latter suspension is used to inoculate each assay tube.

Concentration of folic acid for standard curve—0-2.0 mγ/10 cc. of final medium in arithmetically spaced, graded doses. Samples are added in amounts calculated to yield several points which fall within this assay range.

Sterilization—Autoclaved at 15 lb. pressure for not more than 5-10 min. Longer autoclaving times caramelize the medium and render it unsatisfactory for growth of *L. casei.*

Incubation time and temperature—For turbidimetric use, 16-18 hr. at 37° C. For acidimetric assay, 60-72 hr. at 37° C.

Measurement of response—Turbidimetric or acidimetric. If titration of acid is used, the titrations are best carried out potentiometrically, since the medium is highly buffered, and does not yield a satisfactory end point with bromthymol blue.

Comment—The medium, with folic acid present and the appropriate vitamin omitted, can also be used for assay of riboflavin, pantothenic acid, nicotinic acid and biotin. The chief innovation of the medium lies in the use of an enzymatic digest of casein in place of the acid-hydrolyzed casein of earlier media. Such digests contain one or more unidentified substances, presumably peptides, which greatly stimulate growth of *L.*

casei (183, 184), and are, perhaps, the same as those added to Medium 29 of Teply and Elvehjem with their charcoal-treated peptone. When applied to a variety of materials, the method gave the same results (acidimetrically) as that of Teply and Elvehjem, but was superior to the latter for turbidimetric assays.

All of the ingredients of this medium are present in great excess; if each ingredient is added in one-half the recommended concentration, growth of the test organism is still very luxuriant, and is only slightly less luxuriant than that obtained with the more concentrated medium.

In using the medium, there is sometimes noted a flocculent precipitate following addition of salts C to the medium. On dilution, autoclaving, and cooling, this disappears and does not disturb the assay. If desired, it may be filtered out without damage to the assay medium.

(4) Other Lactic Acid Bacteria Available for Assay of Folic Acid

Requirement for folic acid is distributed somewhat at random among the lactic acid bacteria. Seven of 19 strains of enterococci (which include the common assay organism *S. faecalis* R) required it for growth, but none of 36 strains of *Streptococcus lactis* require it (38, 42). Several lactobacilli, but by no means all, require it, including a large number of strains of *L. casei*, some of *L. delbrueckii* and others (33). At present, no reports of attempted use of organisms other than *L. casei* or *S. faecalis* for assay purposes have been made.

G. VITAMIN B_6

(1) Extraction from Natural Materials

The recognition that several different compounds with vitamin B_6 activity occur naturally, and that these various compounds show different stabilities, and different activities for various test organisms (212), has made it evident that the microbiological assay of this vitamin is not the simple task that it appeared before 1942. At present, the following compounds with vitamin activity for animals are known to occur naturally: pyridoxine, pyridoxal, pyridoxamine (13), pyridoxal phosphate (213), pyridoxamine phosphate (214, 215, 216), and conjugates of pyridoxine of unknown structure in urine (217) and rice bran extract (218). For those microorganisms so far tested, however, pyridoxal phosphate (219) and the conjugated forms of pyridoxine present in rice bran extract and urine (218) show only slight activity prior to hydrolysis. Pyridoxamine phosphate, in contrast, is active (215) or even essential (214) for some organisms, though relatively inactive for others (215). Much of the vitamin B_6 of natural materials is present in these and possibly other bound forms. Hydrolytic treatment preliminary to microbiological assay is, therefore, necessary if all of the vitamin B_6 present is to be released in a measurable form.

In developing extraction procedures for vitamin B_6, release of the vitamin has been followed by early chemical methods (218, 217, 220) or by means of microorganisms such as *Saccharomyces carlsbergensis* (221, 219, 222) and *Neurospora sitophila* (223), for which the 3 known unconjugated forms of vitamin B_6—pyridoxal, pyridoxamine, and pyridoxine—are approximately equally active (225). Consequently, it is not always certain that the ratios of these 3 compounds present in such extracts is a reflection of the ratios in which they were present in the original tissue. Some of the pyridoxal originally present, for example, might interact with amino acids during the extraction procedure to form pyridoxamine (*cf.* 226). In a similar fashion, pyridoxal might be formed from pyridoxamine if the proper carbonyl compounds were present in sufficient amounts (226), or some of the pyridoxal present could be irreversibly inactivated by combination with tryptophan (226). A recent investigation, however, indicates that the hydrolytic procedure recommended below does not produce marked changes in the ratios of the various forms of vitamin B_6 which occur naturally (227).

For many purposes, knowledge of the total amount of vitamin B_6 present is sufficient. For such determinations, extracts prepared by digestion with takadiastase (221), or by heating with 0.04-3 *N* mineral acid in the autoclave for varying lengths of time (219, 220, 221, 222, 223, 224) have been recommended. For many materials, but not all, hydrolysis with low concentrations of acid is superior to use of higher concentrations (221, 219, 222, 228). Originally, this behavior was thought to result from destruction of one form of vitamin B_6 by concentrated mineral acids (228). It is now known, however, that pyridoxal phosphate and pyridoxamine phosphate behave similarly (219), and that prolonged hydrolysis with concentrated acid (1-4 *N*) eventually liberates the same amounts of vitamin B_6 as does 0.055 *N* acid (219, 222, 229). Rubin *et al.* (222) showed that with those materials for which 0.055 *N* acid is the superior extractant, a definite pH optimum for the extraction exists between 1.5 and 2.0. When used as recommended, 0.055 *N* H_2SO_4 or HCl give a pH within this range, usually at about 1.8.

Like riboflavin, vitamin B_6 is readily destroyed by light (229a, 230, 231, 232). The same precautions to avoid light which are used for the assay of riboflavin are effective and should be employed with vitamin B_6..

As indicated earlier, no single extraction procedure is known which will give maximum results with all products. The extraction procedures recommended below are the most effective and generally applicable of those studied to date.

a. Recommended Procedure for Extraction of Vitamin B_6.

(1) *Acid hydrolysis*—In a comparative investigation of the merits of hydrolysis with 0.055 *N* HCl, 2.0 *N* HCl, or takadiastase, the following procedure was found (219) to give maximum values (total vitamin B_6, assayed with *Saccharomyces carlsbergensis* 4228) for all products tested except a rice bran concentrate. Considerably longer periods of heating are required if the autoclave is set at 15 lb. rather than 20 lb. pressure.

A finely divided sample estimated to contain about 2 γ of vitamin B_6 is suspended in 180 cc. of 0.055 *N* HCl. The mixture is autoclaved at 20 lb. pressure for 5 hr., cooled, adjusted to pH 5.0, diluted to a convenient volume, filtered, and aliquots taken for assay.

Rubin *et al.* (222) recommend a very similar procedure, but use H_2SO_4 in place of HCl, and autoclave for 3 hr. at 20 lb. pressure. The 1 hr. autoclaving period originally recommended with 0.055 *N* acid (221) is adequate for some yeast samples, but not for many other materials.

For some plant products, typified by rice bran concentrates and some wheat products, hydrolysis with stronger acid is required. Siegel *et al.* (224) recommended autoclaving at 15 lb. pressure for 30 min. with 2 *N* H_2SO_4. Atkin *et al.* (221) found autoclaving for 1 hr. at 20 lb. pressure with 0.44 *N* H_2SO_4 (180 cc. for a sample containing 2-4 γ of vitamin B_6) effective for these products.

(2) *Enzymatic digestion*—Atkin *et al.* (221) recommend the following procedure. A portion of the sample containing 2-4 γ of vitamin B_6 is suspended in a small quantity of water in a graduated test tube, 0.5 cc. of potassium citrate buffer (pH 5.2: 100 g. potassium citrate monohydrate and 20 g. citric acid/l.) is added, then water to 10 cc. The tube is steamed for 30 min., cooled, and a quantity of clarase equal to the weight of the sample (but not less than 100 mg.) is added. A few drops of benzene are added, the tube is tightly corked, and incubated for 3 days at 37.5° C. or 2 days at 45° C. At the end of the incubation, the tube is steamed for 20 min., the contents diluted suitably and assayed.

With a very similar procedure, Rabinowitz and Snell (219) obtained maximal values for some samples; more often, values were somewhat lower than those found by acid hydrolysis as recommended in (a(1)) above.

(2) Use of Lactic Acid Bacteria for Assay of Vitamin B_6

The lactic acid bacteria are not useful for the estimation of total vitamin B_6, as cultures thus far investigated are unable to utilize pyridoxine

(233, 13, 12, 210, 225) while some appear also unable to utilize pyridoxamine (13, 225). It was this property of these organisms which permitted initial discovery of these new forms of vitamin B_6. The growth-promoting properties of pyridoxine for organisms such as *L. casei* and *S. faecalis* appear to arise from secondary changes, such as oxidation to pyridoxal, or oxidation and transamination to form pyridoxamine, which occur when pyridoxine is autoclaved with culture media (210, 225) or merely is incubated with such media. Such reactions take place to a very small extent only; the microscopic yield of the active compounds accounts for the large amounts of pyridoxine (relative to the amounts of the other vitamins) which are required for lactic acid bacteria. No utilization of pyridoxine, as such, can be demonstrated by either *S. faecalis* (12) or *L. casei* (233), and growth of these organisms will actually fail for lack of vitamin B_6 (pyridoxal or pyridoxamine) in media which contain an excess of pyridoxine (12, 233).

Of the three known forms of vitamin B_6, only pyridoxal is highly active in supporting growth of *L. casei;* pyridoxamine and pyridoxine have negligible activity. For *S. faecalis,* pyridoxal and pyridoxamine are both highly active, while pyridoxine is without activity (13, 225). These facts should permit differential assays for the various forms of vitamin B_6 to be made: Pyridoxal can be determined with *L. casei,* pyridoxal and pyridoxamine with *S. faecalis,* pyridoxal, pyridoxamine and pyridoxine with *Saccharomyces carlsbergensis* or *Neurospora sitophila.* Such differential assays, in conjunction with various chemical treatments, have been carried out and prove the natural occurrence of pyridoxal and pyridoxamine (234, 236).

In their early forms these procedures were cumbersome and inexact. Subsequent investigations have permitted much more accurate determinations of the amounts of pyridoxal and pyridoxamine present in natural materials (227, 219, 215, 235, 236).

(3) Assay Media for Vitamin B_6 and Their Development

It was early recognized (237, 33, 14a) that vitamin B_6, then considered synonymous with pyridoxine, was required for growth of some lactic acid bacteria, and this vitamin was added routinely to semisynthetic media for assay of other nutritive requirements. Once media of known composition were available for organisms which required vitamin B_6, it became possible to omit this vitamin and test for its presence. Media of this nature were developed about 1940-1942 for both *S. faecalis* (206, 207) and *L. casei* (49); their practical use for study of vitamin B_6 awaited availability of concentrates of folic acid free of vitamin B_6. The latter also became available (15) in 1941. The existence of forms

TABLE XII

Composition of Media for Assay of Pyridoxal and Pyridoxamine with Lactic Acid Bacteria

Constituent	31a and b. Snell and Rannefeld, 1945 (225) a. (for *S. faecalis*)	31a and b. Snell and Rannefeld, 1945 (225) b. (for *L. casei*)	31c. Rabinowitz and Snell, 1947 (215) (for *S. faecalis*)	31d. Rabinowitz, Mondy and Snell, 1948 (227) (for *L. casei*)
	Amount/100 cc. of double-strength medium			
Glucose	2.0 g.	2.0 g.	2.0 g.	2.0 g.
Acid-hydrolyzed casein	1.0	1.0	1.0	1.0
Trypsin digest of casein	—	—	—	2.0
Sodium acetate	1.2	1.2	—	1.2[d]
Sodium citrate	—	—	4.0	—
DL-Alanine	—	40 mg.	—	—
L-Asparagine	20 mg.	20	20 mg.	20 mg.
L-Cystine	20	20	80[c]	40
L-Tryptophan	10	10	10	10
Glycine	40	—	40	40
Adenine sulfate	2	2	—	—[e]
Guanine hydrochloride	2	2	—	—
Uracil	2	2	—	—
***p*-Aminobenzoic acid**	20 γ	20 γ	40 γ	40 γ
Biotin	0.08	0.08	0.4	0.4
Calcium pantothenate	40	40	80	80
Folic acid	0.15[a]	0.15[a]	2	2
Nicotinic acid	40	40	80	80
Riboflavin	40	40	80	80
Thiamine chloride	20	20	40	40
Inorganic salts				
Solution B[b]	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
KH$_2$PO$_4$	0.6 g.	0.1 g.	0.6 g.	0.1 g.
K$_2$HPO$_4$	0.6	0.1	0.6	0.1

[a] Recalculated in terms of pure pteroylglutamic acid.

[b] As in Medium 8, Table IV.

[c] The high cystine level is essential. It can be added all before autoclaving, or partially before and partially after (235).

[d] The amount of sodium acetate indicated is in addition to that furnished by the tryptic digest of casein. When prepared as recommended, this latter ingredient supplies about 985 mg. of sodium acetate, which brings the total concentration to slightly over 2% of the double-strength medium.

[e] Addition of purine and pyrimidine bases is optional. With *L. casei*, no difference in response to pyridoxal is noted whether they are present or absent.

of vitamin B_6 other than pyridoxine was announced shortly thereafter, in 1942 (12). The earliest medium used for these assays (12) was identical with that used for folic acid assay (Medium 27, Table XI), except that pyridoxine was omitted and folic acid added. Subsequently (224), the medium was modified for both *S. faecalis* and *L. casei,* and used for preliminary determinations of pyridoxal and pyridoxamine in natural materials. It was recognized that these media (31a and c, Table XII) were not suitable for use in routine assays, and they have been further modified for this purpose, as shown in the table (31b and d). Johnson and coworkers (238) have used the folic acid assay medium of Luckey *et al.* (Medium 28, Table XI) for preliminary assay of vitamin B_6 with *S. faecalis*, by omitting pyridoxine and adding folic acid and salts "B" to the medium. Further details of their procedure are not available; it is, therefore, not included below.

(4) Condensed Summary of Assay Methods for Pyridoxal and Pyridoxamine Employing Lactic Acid Bacteria

a. Pyridoxal.

(1) *Method of Snell and Rannefeld* (225). This procedure used Medium 31b, Table XII, with *L. casei* 7469 as test organism. It was a forerunner of method (2), described below, and, since it was less satisfactory, will not be described further.

(2) *Method of Rabinowitz, Mondy and Snell* (227). Assay organism --*Lactobacillus casei* ATCC No. 7469. Carried as stab cultures by biweekly transfer in Medium b, Table II. At intervals, transfers to the enriched medium of Hunter (239) were made, then, following incubation for 24 hr., the culture was retransferred to Medium b.

Basal Medium—Medium 31d, Table XII. Initial pH 6.8-7.0.

Preparation of acid-hydrolyzed casein for basal medium—50 g. of purified casein (*e.g.*, Labco "Vitamin-free") are refluxed 8-10 hr. with 500 cc. of constant boiling HCl. Excess HCl is removed by concentration to a heavy syrup under reduced pressure. The syrup is dissolved in water, and the concentration repeated. The product is dissolved, adjusted to pH 7.0 with concentrated NaOH solution, diluted to 500 cc., stirred for 15 min. with 5 g. of activated charcoal (*e.g.*, Darco G-60) and filtered. The charcoal treatment is repeated and the resulting solution stored under toluene until used.

Preparation of trypsin-digest of casein for basal medium.—See section F3e p. 404. Certain batches of casein are more acidic than others. If, after dissolving the casein in the bicarbonate, the pH is not at 8.0 or above, it should be brought to 8.0 by addition of NaOH.

Inoculum medium—5 cc. of basal medium, 0.1 γ of pyridoxal hydrochloride and 5 cc. of water per tube. Sterilize and store in the refrigerator.

Inoculum—Incubated at 37° C. for 20-28 hr. Cells are centrifuged out, resuspended in 10 cc. of 0.9% NaCl solution, recentrifuged, and again suspended in 10 cc. of sterile saline. One cc. of this suspension is used to inoculate 100 cc. of the sterile, double strength medium.

Concentration of pyridoxal for standard curve—0, 0.001, 0.002, 0.003, 0.004, 0.005, 0.007 and 0.01 γ of pyridoxal hydrochloride/10 cc. of final medium. Samples are added at levels calculated to furnish amounts of pyridoxal within this range. Neither standard nor samples may be autoclaved with the medium.

Procedure—Water sufficient to make a volume of 5 cc. after addition of samples is added to the assay tubes which are capped and autoclaved at 15 lb. pressure for 10 min. After cooling, the samples are added. These are best sterilized separately, diluted appropriately in sterile water, and added aseptically (*cf.* also 227). The basal medium, previously autoclaved for 10 min. at 15 lb. pressure and cooled, is inoculated, mixed well, and 5 cc. aliquots of this inoculated medium added aseptically to each assay tube.

Incubation time and temperature—22-26 hr. at 37° C. If a large assay has been made, steam for 10 min. to stop growth before determining response.

Measurement of response—Turbidimetric.

Comment—Standard or samples must not be autoclaved with the medium because this treatment transforms a large portion of the pyridoxal present to pyridoxamine by transamination (226, 234). Pyridoxamine and pyridoxine are essentially inactive for the test organism. The enzymatic casein digest is added to this medium to supply "strepogenin" and perhaps other peptides required for rapid growth of the test organism (51). When this is done, the DL-alanine present in medium 31b must be omitted, since in the presence of both D-alanine and enzymatic digest of casein, vitamin B_6 is not required by the test organism (240). Samples which contain D-alanine will thus interfere in the assay. Fortunately, amino acids of the D-series are not widely distributed.

b. Pyridoxal Plus Pyridoxamine.

(1) ***Method of Snell and Rannefeld*** (225). This procedure used medium 31a, Table XII with *Streptococcus faecalis* R, ATCC 8043, as test organism. It has been superseded by the improved method (2), given below, and will not be described further.

(2) *Method of Rabinowitz and Snell* (235).—Assay organism.—*Streptococcus faecalis* R ATCC 8043. Carried as stab culture by monthly transfer in Medium b, Table II. Basal medium.—Medium 31c, Table XII. Initial pH 6.8.

Preparation of acid-hydrolyzed casein for assay medium.—As in method of Rabinowitz *et al.* for pyridoxal, outlined in section a (2) above.

Inoculum medium—5 cc. of basal medium, 0.1 γ of pyridoxal hydrochloride and 5 cc. of distilled water per tube. Sterilized by autoclaving and stored until needed.

Inoculum—Incubated 20-24 hr. at 30° C. Cells centrifuged, washed in 10 cc. of sterile saline, recentrifuged, and resuspended in 10 cc. of sterile saline. One drop of this heavy suspension is used to inoculate each assay tube.

Concentration of pyridoxal and pyridoxamine for standard curves—0, 0.0005, 0.00075, 0.001, 0.0015, 0.002, 0.003, 0.004, 0.01 γ/10 cc. of final medium. Samples should contain between 0.0005 and 0.003 γ of these compounds.

Procedure—As described under the assay for pyridoxal, section a (2) above. In both assays, it has frequently proved possible to use unsterilized samples, diluted appropriately with sterile water for assay without introducing contamination.

Incubation time and temperature—22-24 hr. at 30° C.

Measurement of response—Turbidimetric.

Comment—The shapes of the standard curves obtained with pyridoxal and with pyridoxamine frequently differ. Where the method is run in conjunction with the pyridoxal assay with *L. casei* to provide a differential assay for both pyridoxal and pyridoxamine, both standards must be run. The value for the pyridoxal content of the sample, determined with *L. casei,* is then converted to its equivalent of pyridoxamine for *S. faecalis,* and subtracted from the total value found with *S. faecalis* against the pyridoxamine standard to give the pyridoxamine content of the sample (*cf.* 236). Alternatively, and somewhat more accurately, the pyridoxal of the sample can be destroyed by treatment with acetone and alkali (234), when the sample can be assayed directly for pyridoxamine. Pyridoxamine phosphate is active for this organism, and unless completely hydrolyzed will give rise to "drift" in the assay values. D-alanine is active in place of vitamin B_6 for this organism, as it is for *L. casei,* and will interfere when present in samples in large amounts.

The presence of sufficient cystine in the medium is of critical importance to this assay procedure. It may be added in excess to the medium as recommended above, but autoclaving must not be too prolonged (not

$>$ 10 min.) or it is destroyed. In the original method (235) a sterile solution of cystine was added to the basal medium after autoclaving to supply a level of 3 mg./10 cc. of the final medium.

(5) Other Lactic Acid Bacteria Available for Assay of Vitamin B_6

A partial survey of organisms of this group reveals that a very large number of them require vitamin B_6 for growth (38, 42, 225, 241). In the few instances examined, pyridoxine showed very low activity compared with pyridoxamine or pyridoxal, but more investigation is needed of this point. Rabinowitz and Snell (215) compared the utility of 3 additional strains of *Streptococcus faecalis* as assay organisms for pyridoxal and pyridoxamine with that of *S. faecalis* R. All organisms gave the same results, and there were no indications of superiority of one over another for this purpose.

H. *p*-AMINOBENZOIC ACID

(1) Extraction from Natural Materials

Since *p*-aminobenzoic acid was identified as a component of the folic acid molecule (200), its status as a vitamin for higher animals, never unquestioned, needs re-examination. There is no question, however, that *p*-aminobenzoic acid is an essential vitamin or growth-factor for some microorganisms, *e.g.*, *Acetobacter suboxydans* (242), *Clostridium acetobutylicum* (11), mutant strains of *Neurospora* (243) and of *Escherichia coli* (244), as well as for some lactic acid bacteria to be considered below. In this role, folic acid does not replace it for organisms so far examined (*cf.* 244).

This presents an unsolved problem to those who wish to determine *p*-aminobenzoic acid. This substance is known to occur naturally in bound forms (245, 246, 247), one of which, a *p*-aminobenzoylpolyglutamylglutamic acid (246) has been isolated from yeast. Procedures designed to liberate *p*-aminobenzoic acid from such bound forms, however, may also liberate a certain amount of that present in folic acid. At present, the effective limits of action of various extraction procedures for *p*-aminobenzoic acid are not known. Substantially different procedures have been used by various investigators. Lewis (40) found that dilute solutions of pure *p*-aminobenzoic acid are stable to autoclaving at 10 lb. steam pressure for 30 min. with 0.1 *N* NaOH; 1 *N* NaOH gave 10% destruction, 10 *N* NaOH gave 50% inactivation. The pure compound was approximately 30% inactivated under the same conditions by treatment with H_2SO_4 or HCl at concentrations varying from 0.1 to 10 *N*.

In many cases, alkaline autoclaving of natural extracts increased the amount of *p*-aminobenzoic acid found, but no procedure for extraction was recommended. All of his assays were made with *Lactobacillus arabinosus* (40). Thompson *et al.* (248), using a mutant culture of *Neurospora crassa* as test organism, found approximately 15% destruction of pure *p*-aminobenzoic acid by hydrolysis with 6 *N* H_2SO_4 but, despite this destruction, this treatment gave maximal figures for the *p*-aminobenzoic acid content of natural materials. They add 1 g. of finely divided sample to 5 cc. of 6 *N* H_2SO_4, autoclave for 1 hr. at 15 lb. pressure, cool, nearly neutralize the acid with $BaCO_3$, remove all insoluble materials ($BaSO_4$ and undigested sample) by filtration, and assay aliquots of the extract. Pennington (41) found a similar procedure effective for *Leuconostoc mesenteroides* but autoclaves for only 15 min.

A radically different treatment was found to yield maximum values by Lampen and Peterson (249). They found acid hydrolysis superior to enzymatic digestion procedures, but obtained much the highest results by autoclaving with 5 *N* NaOH at 75-80 lb. pressure for 1 hr. Similarly high values could be obtained by autoclaving at only 15 lb. pressure, but 8-12 hr. hydrolysis periods were required. Good recoveries of added *p*-aminobenzoic acid were obtained. *Clostridium acetobutylicum* was used as test organism (249). Pennington, however, using *Leuconostoc mesenteroides,* found destruction of the *p*-aminobenzoic acid of natural materials by alkaline hydrolysis, although the pure chemical was stable under similar conditions (41). The discrepancy has not been resolved.

(2) Assay Media for Determining p-Aminobenzoic Acid with Lactic Acid Bacteria, and Their Development

Only two lactic acid bacteria, *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* P-60 have been reported to require *p*-aminobenzoic acid for growth. Both organisms have comparatively simple vitamin requirements (see Table I) and the other requirements for their growth, with the exception of *p*-aminobenzoic acid, are met by such media as No. 8, Table IV, when supplemented with nicotinic acid. On media such as this, the quantitative requirement for *p*-aminobenzoic acid is extremely small.[36] Indeed, Medium 8 as ordinarily prepared usually can be used successfully with *L. arabinosus* for assay of nicotinic acid without the addition of any *p*-aminobenzoic acid. Two reasons for this behavior are now known: (1) the extremely small amounts of *p*-aminobenzoic acid required for growth are quite often present as a contaminant in other ingredients of the medium, particularly the casein hydrolyzate; (2) it seems to be quite definitely established that strains of *L. arabinosus* 17-5 exist which do not require *p*-aminobenzoic acid for growth, and strains which require this vitamin can give rise to the non-requiring type spontaneously (41). Whether or not the latter difficulty will arise in the use of *Leuconostoc mesenteroides* for assay cannot be said at present.

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[36] If the purine and pyrimidine bases are eliminated from the medium, *p*-aminobenzoic acid effectively replaces them for *L. arabinosus,* and considerably larger amounts of the vitamin are required for growth. This is true even with cultures which cannot be demonstrated to require *p*-aminobenzoic acid when purine bases are present in the medium (250). The finding has not been applied to assay of *p*-aminobenzoic acid, since presence of purine bases would presumably interfere.

(3) Condensed Summary of Assay Methods for p-Aminobenzoic Acid Which Employ Lactic Acid Bacteria

a. Method of Lewis (40).

Assay organism—*Lactobacillus arabinosus* 17-5 ATCC No. 8014. Precautions must be taken to make certain that the culture received requires *p*-aminobenzoic acid. Carried as stab cultures by monthly transfer in Medium "b," Table II.

Basal medium—Medium 8, Table IV, supplemented with 80 γ nicotinic acid/100 cc. of double strength medium. Initial pH 6.8.

Preparation of hydrolyzed casein for basal medium—100 g. of vitamin-free casein are hydrolyzed under reflux for 9 hr. with 1000 cc. of 6 *N* HCl. To remove most of the HCl the mixture is concentrated *in vacuo* to a thick syrup, dissolved in water and reconcentrated. The syrup is diluted to 900 cc., adjusted to pH 3.0 with concentrated NaOH, and stirred for 30 min. with 9 g. of Norit A (Pfanstiehl). The activated carbon is removed by filtration through a Whatman No. 3 filter paper, and the Norit treatment and filtration repeated. The filtrate is adjusted to pH 6.8 with concentrated NaOH, diluted to 1000 cc., and stored in the refrigerator. On standing, precipitation of tyrosine occurs. No difference in results has been noticed whether this precipitate is discarded or left in the basal medium.

Inoculum medium—5 cc. of basal medium, 1 mγ *p*-aminobenzoic acid, and 5 cc. of water per tube. Plugged with cotton and sterilized by autoclaving, and stored until use.

Inoculum—Incubated at 30° C. for 24 hr. Cells are centrifuged and resuspended in the original volume of sterile 0.9% NaCl solution. Each assay tube is inoculated with 1 drop (equivalent to approximately 0.04 cc.) of this suspension.

Concentration of *p*-aminobenzoic acid for standard curve—0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 mγ/10 cc. of final medium. Samples added in amounts calculated to furnish *p*-aminobenzoic acid within this assay range.

Sterilization—Autoclaved at 15 lb. pressure for 10-15 min.

Incubation time and temperature—3 days at 30° C.

Measurement of response—Titration with 0.1 *N* NaOH, with bromthymol blue as indicator.

Comment—A great deal of trouble has been experienced by workers attempting to use this procedure, for reasons described in Section 2 above. Great care is required to prevent contamination of ingredients of the culture medium with *p*-aminobenzoic acid. When conditions are right, the method appears very satisfactory, and as accurate as any of the better microbiological assays.

b. Method of Pennington (41). The basal medium and methodology are exactly similar to those followed by Lewis, and described above, with the following exceptions:

Assay organism—*Leuconostoc mesenteroides* P-60 ATCC No. 8042.

Concentration of *p*-aminobenzoic acid for standard curve—0-1 mγ /10 cc. of final medium.

Incubation time and temperature—15 hr. at 37° C.

Measurement of response—Turbidimetric.

Comment—The assay has not been widely applied as yet. Its weaknesses, if any, are, therefore, not yet apparent. Comparative data presented with the original paper showed close agreement in results of assay of identical extracts by this procedure, and by the method of Lewis, summarized above.

I. VITAMIN B_{12}

The isolation of vitamin B_{12}, the newest of the vitamins, was materially aided by use of a bacterial test discovered by Shorb (251) which employs *Lactobacillus lactis* Dorner (ATCC 8000) as the test organism (19, 20). Details of the assay have not been described, and attempts to carry it out by conventional techniques have generally failed. The difficulties here are perhaps connected with the fact that the vitamin is not required in the presence of thymidine (69, 252, 252a) or of ascorbic acid (low oxidation-reduction potential?) (69, 252a). The vitamin is unavailable in pure form at the time of writing. A considerable number of additional lactic acid bacteria are known which require thymidine in the absence of vitamin B_{12} (253, 252a), and for which vitamin B_{12} replaces thymidine (unpublished data). Ascorbic acid and desoxyribosides of guanine, hypoxanthine, adenine and cytosine also are active for many of these organisms (252a). No procedure for the specific assay of vitamin B_{12} can be presented at the present time. Additional assay organisms will undoubtedly become available from this group.

J. ASSAY METHODS APPLICABLE TO SEVERAL VITAMINS

Cursory examination of the basal media for assay of the various vitamins given in Tables IV-XII above will reveal the great similarity of many of them. Only 3 different organisms, *Lactobacillus arabinosus*, *Lactobacillus casei* and *Streptococcus faecalis*, have been widely used for assay; each of these organisms requires several vitamins for growth (Table I). It should, therefore, be feasible to develop a single medium for any given organism which, by omitting the single vitamin to be determined, could be used for determination of any vitamin required by that organism. This possibility has been pointed out by various authors, but the large number of assays necessary to prove the applicability of such general methods to all of the vitamins have not been carried out.

(1) Use of Lactobacillus arabinosus for Determination of Biotin, Nicotinic Acid, and Pantothenic Acid

Snell and Wright (48), in 1941, pointed out that their method for nicotinic acid, which utilizes *Lactobacillus arabinosus* as the test organism, could be applied also to the determination of pantothenic acid and biotin, simply by adding an excess (80 γ/100 cc. of double strength medium) of nicotinic acid to the medium (Medium 8, Table IV), and omitting either biotin or pantothenic acid. This can be done equally well with the various modifications of this original medium suggested for assay of nicotinic acid such as that of Krehl *et al.* (50), Barton-Wright (121), Lawrence *et al.* (44), or the Collaborative USP-AOAC medium (118)—media 9, 10, 11, and 12 of Table IV, respectively. Skeggs and Wright (156) and Wright and Skeggs (164) have modified the medium slightly for assay of pantothenic acid (Medium 21, Table VIII) and of biotin (Medium 25, Table IX). The same media can be used for nicotinic acid, supplemented, if desired, by additional sodium acetate to permit higher acid production. In the author's experience, such a common procedure for these three vitamins is just as satisfactory, and much simpler, than maintaining slightly different media and procedures for each one.

(2) Use of Lactobacillus casei for Determination of Biotin, Folic Acid, Nicotinic Acid, Pantothenic Acid, Riboflavin, and Vitamin B_6

a. Method of Landy and Dicken (49). These authors, in 1942, suggested a generalized procedure for these 6 vitamins, using *Lactobacillus casei* as test organism. Their medium has been given in connection with determination of riboflavin (Medium 4, Table IIIb) and of biotin (Medium 23, Table IX). The procedure is as follows:

Assay organism—*Lactobacillus casei,* ATCC No. 8014. Carried by monthly transfer in Medium "b," Table II.

Basal medium—Medium 23, Table IX, with the proper vitamin omitted and biotin added (where necessary) as in Medium 4, Table IIIb.

Preparation of hydrolyzed casein for basal medium—To one liter of distilled water at 50° C., 100 g. of casein are added gradually with stirring. After stirring 30 min., 3 g. of $NaHCO_3$ are added, and the stirring continued until the casein is dissolved. More water is sometimes required. When solution is complete, the pH is adjusted to 4.6 with 10% HCl. The casein precipitate is allowed to settle and is removed by filtration. The reprecipitation is repeated several times, depending on the purity of the original casein. 100 g. (dry wt.) of the reprecipitated casein are hydrolyzed with 500 cc. of 25% H_2SO_4 for 10 hr. at 15 lb. pressure. The H_2SO_4 is neutralized with $Ba(OH)_2$, and the $BaSO_4$ removed by filtration. The resulting casein hydrolyzate solution is adjusted to a total solids content of 10%, acidified to pH 3 and treated with activated charcoal (Nuchar xxx), 1 g. for each 10 g. of casein. The mixture is stirred for 1 hr. and filtered through Filter-Cel. The resulting solution may be preserved with toluene until used.

Inoculum medium—Yeast dextrose broth (1% yeast extract, 1% glucose). Sterilized as usual and held until used.

Inoculum—Incubated 24 hr. at 37° C.; cells centrifuged out, resuspended in an equal volume of sterile 0.85% NaCl solution, recentrifuged, the sediment resuspended in 4 cc. of saline, and 1 cc. of this suspension added to 85 cc. of sterile saline. One-tenth cc. of this diluted suspension is used to inoculate each assay tube.

Concentration of vitamin required for standard curve—

Riboflavin: 0-0.4 γ/10 cc. of final medium.
Folic acid: 0-1.5 mγ/10 cc. of final medium.
Nicotinic acid: 0-0.8 γ/10 cc. of final medium.
Pantothenic acid: 0-0.2 γ/10 cc. of final medium.
Biotin: 0-2 mγ/10 cc. of final medium.
Pyridoxine: 0-0.5γ/10 cc. of medium. (Pyridoxine actually is inactive; its activity depends on autoclaving time. Later information (225) indicates pyridoxal would be active in the range 0-.01 γ/10 cc. of medium. See section on vitamin B_6 above.)

Sterilization—15 lb. pressure for 15 min.

Incubation time and temperature—72 hr. at 37.5° C.

Measurement of response—Titration of acid produced with 0.1 *N*

NaOH, with bromthymol blue as indicator. Electrometric titration is also recommended.

Comment—This medium has been rather extensively used for the assay of folic acid. For other vitamins, its use has not been widespread, since it is known to be lacking in substances which, though not essential for growth, greatly stimulate the rate of growth and permit much heavier growth (183, 184, 254). Despite this fact, figures obtained with it usually agree fairly well with those obtained on identical extracts by other methods. The method cannot be used for determination of pyridoxine, as originally claimed, for reasons discussed in the section on vitamin B_6, above.

The most effective of the stimulatory substances lacking in this medium are present in enzymatic digests of casein and of other proteins (184). They have been added to the medium of Roberts and Snell, considered below, by substituting an enzymatic digest of casein for the acid hydrolyzate of casein used in earlier media.

b. Method of Roberts and Snell (51). This medium, which has been considered under riboflavin (Medium 7, Table IIIb) and folic acid (Medium 30, Table XI) was devised to supply the major stimulatory factors missing in the Landy and Dicken medium, by substituting an enzymatic digest of casein for the acid-hydrolyzed casein of previous media. Its glucose content, buffer capacity, and content of essential nutrients, have also been increased, to permit faster growth and higher acid production. The procedure for using it has been summarized under folic acid. For other vitamins, folic acid is added to Medium 30 at a level of 0.4 γ/100 cc. of double strength medium, and the vitamin to be determined is omitted. In all other details, the procedure is the same as that described earlier. The assay ranges for the various vitamins are as follow:

Biotin: 0-1.2 mγ/10 cc. of final medium.
Folic acid: 0-1.2 mγ/10 cc. of final medium.
Nicotinic acid: 0-0.2 γ/10 cc. of final medium.
Pantothenic acid: 0-0.2 γ/10 cc. of final medium.
Riboflavin: 0-0.2 γ/10 cc. of final medium.

Pyridoxal cannot be determined with the medium as originally described, since it contains sufficient vitamin B_6 (as an impurity in the casein, not removed by the charcoal treatments) to mask the response to additional vitamin.

The medium is too recent to have been used widely. Failures with the medium so far reported to the author have resulted from (a) incomplete suspension of casein, with consequent poor digestion, (b) use of too prolonged sterilization times, with consequent damage to the growth-promoting properties of the medium, or (c) failure to obtain complete removal of vitamin impurities from the enzymatic casein digests by the recommended charcoal treatment, as evidenced by very high blanks in the various assays. This difficulty is not usually met if Labco "Vitamin-free" casein is used; it can, however, be minimized by carrying out the second charcoal adsorption at pH 3.0 rather than 3.8, by extracting the casein with alcohol (50) before digestion, or by repeated reprecipitations of the casein preliminary to digestion, as recommended by Landy and Dicken (49). The casein digest can also be employed at one-half the recommended level with very little diminution in the growth obtained.

The medium has not been applied widely enough to evaluate the results obtained with it.

(3) Use of Streptococcus faecalis R for Determination of Biotin, Folic Acid, Nicotinic Acid, Pantothenic Acid, and Vitamin B_6

No general methods using this organism have been fully investigated. However, excellent standard curves for each of these vitamins have been obtained (235) in Medium 31c, Table XII, which is that recommended for determination of pyridoxamine, and there is no known reason why this medium and general procedure should not be satisfactory for these other vitamins as well.

III. Methods Utilizing Yeasts as Assay Organisms

1. The Nutritive Requirements of Yeasts Used in Assay Work

Investigation of the nutritional requirements of microorganisms was first begun with yeast. Von Liebig, in 1871, observed that a certain yeast did not grow properly in a synthetic culture medium unless stimulating materials from cell extracts also were present. These observations were overshadowed by those of Pasteur, who showed, also correctly, that yeasts can assimilate nitrogen from ammonium salts as the sole nitrogen source. Historically, the two observations were considered contradictory, but the discrepancy is easily resolvable in terms of today's knowledge. Some yeasts do grow on mixtures of inorganic salts and a fermentable carbohydrate, and many more can be trained to do so by proper cultural procedures (*e.g.*, 255), including use of large inocula, as practiced by Pasteur.

The present phase of nutritional investigations with yeast began in

1901, when Wildiers showed (256) that small inocula of yeasts added to a chemically defined medium failed to grow, but that addition of minute amounts of yeast extract permitted growth. The active material in the yeast extract was called "bios" and several of its properties were determined. Wildiers expressed the hope that the name "bios" would soon be replaced by a chemical name for the active substance. Forty-odd years later, this has at last become possible.

The large amount of work initiated by Wildiers observations (reviews: 257, 258, 259, 260) culminated in the identification of inositol (3), thiamine (261), biotin (8), pantothenic acid (4, 5) or its cleavage product, β-alanine (262), vitamin B_6 (15, 263, 264), and nicotinic acid (265, 266, 267), as essential or stimulatory factors for growth of various yeasts. Different species or strains of yeast require different numbers and different combinations of these six vitamins for growth; this phase of the problem has been extensively investigated with a large number of different strains, species, and genera of yeasts (268, 267, 269, 270, 271, 272). As an example, the extensive work of Burkholder, McVeigh and Moyer (267) may be cited. They investigated 163 strains of yeasts belonging in 110 named species and varieties. Of these, 86 of the named kinds required one or more of the six vitamins mentioned above, as follows: biotin, 78; thiamine, 33;[87] pantothenic acid, 30; inositol, 15; nicotinic acid, 13; vitamin B_6, 13. Some species required no vitamins at all (*e.g.*, *Hansenula lambica*), others required all of the six vitamins (*e.g.*, *Kloeckera brevis*); between these two extremes, other species required from one to five vitamins in 18 different combinations.

These six vitamins, therefore, satisfy the vitamin requirements of the great majority of yeasts. Robbins and Ma, however, have found a single yeast species, *Rhodotorula aurantiaca,* which requires *p*-aminobenzoic acid for growth (273), and other species, uninvestigated as yet, may be found with additional requirements. No yeasts have yet been found which require external sources of riboflavin or of folic acid.

In the presence of an adequate supply of the vitamins of the "bios complex," inorganic salts, and a fermentable carbohydrate, all yeasts tested are able to assimilate nitrogen from ammonium salts, urea, asparagine, or peptone (amino acids) as the sole nitrogen source for growth (274). Ammonium salts are the most commonly used nitrogen source for yeast growth. No yeasts are known which require external sources of the amino acids; however, addition of amino acids to "complete" media not containing them often improves growth considerably (275, 276, 277, 267).

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[87] The thiamine nutrition of yeasts is complex. Some require molecular thiamine, others can grow if supplied either the intact thiamine molecule or the pyrimidine and thiazole portions of it; some require the pyrimidine half of the molecule only, others the thiazole part only.

Although yeast will grow anaerobically, its growth is very much favored by aerobic conditions. To supply the oxygen helpful for achieving heavy growth, the cultural conditions used for assays differ significantly from those used with lactic acid bacteria. If the yeast is to be grown without shaking, it is often cultured in flasks, or in large test tubes, where the ratio of surface to volume of medium will be high. Even more satisfactory, from the standpoint of securing heavy growth, is the common practice of shaking the culture vessels during growth in such a manner that continuous aeration is assured. For the same reason, stock cultures are carried on agar slants rather than as stab cultures.

Like other fungi, yeasts grow over a wide range of hydrogen ion concentrations. Ordinarily, the initial pH of assay media is about 5.0.

2. Assay Methods for Individual Vitamins

Inositol excepted, it will be unnecessary to cite extraction procedures for the various vitamins mentioned below, since these have been considered in connection with assays utilizing lactic acid bacteria. It is not always certain, of course, that a procedure which renders a vitamin wholly available for one test organism will do so for another; in general, however, and in keeping with its less exacting nutrition, yeast is more versatile with respect to the forms of a vitamin which it can utilize than are the lactic acid bacteria; if extraction for the latter group of organisms is complete, it will almost certainly be so for yeast also.

By far the simplest and most accurate method for following yeast growth quantitatively is to determine the turbidity of cultures with some type of turbidimeter or nephelometer. Turbidimetry, employing commercially available photoelectric colorimeters (278) or specially constructed apparatus (28) is simplest, and is very widely used. Details of the method have been treated by Williams, McAlister and Roehm (28), Thorne (279, 280) and by Atkin, Schultz and Frey (278). In general, the procedures are identical with those discussed earlier in considering turbidimetric assays with lactic acid bacteria.

Since the techniques of assay with yeast differ significantly from those used with lactic acid bacteria, the procedures for several yeast methods (biotin and pantothenic acid) will be given in full. Subsequently, additional methods will be given in more summary fashion, and for omissions of technique, the methods given in more detail can be studied.

TABLE XIII

Composition of Assay Media for Biotin

Constituent	Medium No. 32. Snell, Eakin and Williams 1940 (162, 281)	33. Hertz, 1943 (277)	34. Atkin, Williams, Schultz, and Frey, 1944 (282)
	Amount/100 cc. of basal medium[a]		
Glucose	—	—	10 g.
Sucrose	2.0 g.	3.4 g.	—
Casein, acid-hydrolyzed, charcoal-treated	—	0.1 g.	1.0 g.
Potassium citrate, monohydrate	—	—	1.0 g.
Citric acid, monohydrate	—	—	200 mg.
L-Aspartic acid	10 mg.	17 mg.	—
$(NH_4)_2SO_4$	300 mg.	500 mg.	750 mg.
KH_2PO_4	200 mg.	340 mg.	110 mg.
KCl	—	—	85 mg.
$MgSO_4.7H_2O$	25 mg.	45 mg.	25 mg.
$CaCl_2.2H_2O$	25 mg.	45 mg.	25 mg.
$MnCl_2$	100 γ	180 γ	500 γ[b]
$FeCl_3$	50 γ	90 γ	500 γ
H_3BO_3	100 γ	180 γ	—
$ZnSO_4$	100 γ	180 γ	—
$TlCl_3$	100 γ	180 γ	—
$CuSO_4.5H_2O$	10 γ	18 γ	—
KI	10 γ	18 γ	—
Inositol	500 γ	900 γ	5000 γ
β-Alanine	50 γ	—	—
Calcium pantothenate	—	50 γ	500 γ
Pyridoxine hydrochloride	2 γ	100 γ	50 γ
Thiamine chloride	2 γ	100 γ	50 γ

[a] Media 32 and 33 are much more similar than they appear, since the former is only 1.2 times its final concentration, while the latter is twice its final concentration. Medium 34 is also twice its final concentration.

[b] $MnSO_4$ was used instead of $MnCl_2$.

A. BIOTIN

(1) *Assay Media and Their Preparation*

The composition of media thus far suggested for assay of biotin is given in Table XIII. In preparing such media it is convenient to have stock solutions of the stable ingredients which can be mixed to prepare the final medium. For example, a relatively concentrated stock solution containing the trace mineral elements (from H_3BO_3 to KI) in the proper ratio is convenient in preparing Medium 32, Table XIII. This scheme was followed also in preparing media for assays with lactic acid bacteria; for illustrative techniques, reference can be made to assays for riboflavin and nicotinic acid in that section. The entire mixed medium is often stable for long periods if contamination is prevented by intermittent steaming or by refrigeration. The mixed medium 32, for example, is stable for months under these conditions.

(2) *The Assay Methods*

The original microbiological method for biotin was that of Kögl and Tönnis (8). This important test resulted in the discovery, concentration, and first isolation of this vitamin. The assay involves the growth response of a heavy seeding of "Rasse M" yeast during a 5 hr. growth period. The method is not included below because the yeast strain is not available in this country, and the method has not been investigated from the standpoint of quantitative applicability to crude materials.

a. Method of Snell, Eakin and Williams (162).

Assay organism—*Saccharomyces cerevisiae* F.B.[38] ATCC No. 7754. Carried as slant cultures on molasses agar (Hawaiian cane blackstrap molasses 16.5 g., $NH_4H_2PO_4$ 0.6 g., agar 6.5 g., water 500 cc.). Transferred at weekly intervals. After transfer, cultures are incubated at 30° C. for 24-48 hr., then held in the refrigerator until the next transfer.

Basal medium—Medium 32, Table XIII. Initial pH, usually 3.8-4.0.

Procedure—Graded amounts of standard biotin (0-0.2 mγ) and of samples are pipetted into a series of 50 cc. Erlenmeyer flasks. The volume of these addenda should not surpass 2 cc.[39] The volume in all flasks is adjusted to 2 cc. with water. All flasks are sterilized at 100° C. by running live steam through the autoclave for 5 min. At the same time, a volume of basal medium sufficient for the assay plus a small additional volume (25-30 cc.), and pipettes required later, are similarly pasteurized. It is not necessary to plug or cover the assay flasks.[40] After cooling, a small amount of yeast is removed from a slant culture of the test organism with a bacteriological loop, and suspended uniformly in a 20-25 cc. aliquot of the basal medium. The optical density of this suspension is determined in the photoelectric colorimeter, and the weight of moist yeast/cc. determined by reference to a previously constructed standard curve, relating optical densities to weight of moist yeast.[41] A volume of this inoculum suspension sufficient to furnish 0.02 mg. of moist yeast for each 10 cc. of basal medium is then transferred to the flask containing the basal medium, which is then shaken to uniformly distribute the cells. Ten cc. of this inoculated basal medium are then pipetted into each assay flask. These are then set, unstoppered and without agitation, in an incubator at 30° C. for the growth period of approximately 16 hr. At this time, growth is stopped by diluting the contents of each flask with 10 cc. of saturated aqueous chlorothymol,[42] and the flasks shaken mechanically for 3-5 min. The density of yeast growth in each flask is determined in the photoelectric colorimeter. A standard curve is then constructed by plotting the growth response (as galvanometer readings or optical densities) against the concentration of biotin. The biotin content of the sample flasks is determined by interpolation on the standard curve, and the final biotin content of the sample calculated. This phase of the procedure is exactly similar to that discussed in an earlier section of this chapter (p. 350).

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[38] Fleischmann Baker's.

[39] If one wishes to use larger volumes of standard or sample, the basal medium can be prepared in a more concentrated form and used in sufficient amount later to bring the final concentration of ingredients to the proper level.

Comment—When used with a sensitive turbidimeter of the type used in developing the procedure (28), the method described above is one of the most precise microbiological methods with which the author has had experience. The maximum growth obtained is not great, however, and the range of galvanometer readings obtained between the "blank" flask and the maximum dose is too small to permit maximum accuracy when instruments such as the Evelyn colorimeter are used. The medium has subsequently been modified by Hertz to permit greater growth (Medium 33, Table XIII) and satisfactory use with less sensitive measuring instruments.

When large numbers of assays must be carried out, the use of Erlenmeyer flasks as cultural vessels is somewhat cumbersome. The above method may also be employed with ordinary bacteriological test tubes as cultural vessels. Larger size test tubes are even more satisfactory. In this modification (162) the scale of the assay has been cut in half: standard and supplements are added with a maximum of 1 cc. of water, all tubes are diluted to 1 cc., and 5 cc. of the inoculated basal medium are added. In all other details, the procedure is identical with that described above. For precise work, the author considers the unmodified procedure more satisfactory.

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[40] In the short growth period used for assay, contamination does not occur.

[41] To construct such a curve, a pound cake of pressed yeast is purchased, a known weight suspended in water, and the optical densities of appropriate dilutions determined. Optical densities are then plotted against the weight of suspended yeast/cc.

[42] This procedure is unnecessary unless the assay is large and considerable time is necessary to read the final results. An alternative way to stop growth is to steam all flasks for 5 min. in the autoclave.

In its response to compounds related to biotin, yeast is less specific than are the lactic acid bacteria used for biotin assay. Thus, desthiobiotin, the diaminocarboxylic acid derived from biotin by breaking the ureido ring, biotin sulfoxide, and some other derived products, are utilized by yeast with either full or reduced efficiency, whereas each of these products is inactive for lactic acid bacteria (See p. 389 for references). Whether or not such substances occur naturally is not known. Burk and Winzler (174) have claimed that many products contain avidin-uncombinable substances which stimulate yeast growth under conditions of the biotin assay, but Chu and Williams (175) were unable to confirm this finding when the original testing procedure was used, except in the case of certain urine samples. Hertz (277) also reports disturbing effects from urines and some other samples with the above-described method, most of which presumably are eliminated in his modification described below.

b. Method of Hertz (277).

Assay organism—*Saccharomyces cerevisiae* FB ATCC No. 7754. Carried as slant cultures at room temperature on Sabboraud's agar (peptone 10 g., glucose 40 g., agar 15 g., water to 1 liter), with transfers at least every 3 days.

Basal medium—Medium 33, Table XIII. Initial pH, usually 3.8-4.0.

Preparation of hydrolyzed casein for basal medium—Method employed not stated. However, the procedure described on p. 364 will yield a product suitably free of biotin.

Procedure—The test is carried out in calibrated photometer tubes, 18 × 2 cm. In each culture tube 5 cc. of the basal medium are added to variable portions (not over 5 cc.) of the test material. The assay range for pure biotin is 0-0.4 mγ/10 cc. The final volume is then diluted to 10 cc. with distilled water. The test is sterilized by heating at 100° C. for 5 min. in flowing steam.[43]

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[43] This follows the original procedure of Snell, Eakin and Williams. No mention of method of sterilization is made by Hertz.

Inoculation is with a 24 hr. culture of the assay organism grown at 37° C. in the basal medium containing about 0.5 mγ of biotin/10 cc. This culture is centrifuged, the sedimented cells washed twice with normal saline, and the concentration of the final suspension adjusted turbidimetrically to contain 1 mg. of moist yeast per cc. 0.05 cc. of this suspension is used to inoculate each assay tube.

The inoculated culture tubes are incubated without plugs for 18-22 hr. at 37° C. in a forced draught incubator. Growth response is determined turbidimetrically.

Comment—(*Cf.* also comment to method of Snell, Eakin and Williams above). Recovery tests for biotin added to crude samples ranged between 90 and 100%; successive dilutions of unknown material check within 5%. The chief advantage of the method is a wider working range, which facilitates biotin determinations in samples containing unknown amounts of this vitamin. The author's claim that the modified method has increased specificity over the original method due to the elimination of the nonspecific stimulation which may result from the amino acid content of samples, should be discounted, since curves presented with the modified method show that no such stimulation results in the assay range used with the original method.

c. *Method of Atkin, Williams, Schultz and Frey (282).*

Assay organism and inoculum—*Saccharomyces cerevisiae* No. 188, obtainable from the Fleischmann Laboratories,[44] is carried on Difco malt agar slants. A slant is incubated at 30° C. for 24 hr., and is then transferred to the refrigerator for not more than 2 weeks. To prepare inoculum for assay, a fresh transfer to malt agar is prepared 24 hr. in advance, and is incubated at 30° C. for 24 hr. A quantity of the fresh yeast is removed with a sterile wire loop to 10 cc. of sterile 0.9% NaCl, and suspended thoroughly. The transmission is read in the colorimeter, and the concentration of yeast adjusted to 1 mg./cc. by addition of saline or yeast, as required. The calibration is based on a known suspension of moist baker's yeast. Five cc. of this suspension are then added to 45 cc. of sterile saline in an Erlenmeyer flask. The final suspension thus contains 0.1 mg. of moist yeast/cc. and is ready for use as inoculum.

Basal medium—Medium 34, Table XIII. The medium is prepared by mixing a series of stock solutions of the following composition:

(1) Sugar and salts solution. One liter contains 200 g. C.P. dextrose,

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[44] Many other biotin-requiring yeasts, such as *Saccharomyces carlsbergensis* 4228, can also be used with the same medium, apparently interchangeably.

2.2 g. KH_2PO_4, 1.7 g. KCl, 0.5 g. $CaCl_2.2H_2O$, 0.5 g. $MgSO_4.7H_2O$, 0.01 g. $FeCl_3$, and 0.01 g. $MnSO_4$.

(2) Potassium citrate buffer. One liter contains 100 g. potassium citrate monohydrate and 20 g. citric acid monohydrate.

(3) Thiamine solution. 10 γ thiamine chloride/cc.

(4) Pyridoxine solution. 10 γ pyridoxine hydrochloride/cc.

(5) Inositol solution. 1 mg. of inositol/cc.

(6) Calcium pantothenate solution. 100 γ of calcium pantothenate/cc.

(7) Ammonium sulfate solution. 150 mg. of $(NH_4)_2SO_4$/cc.

(8) Casein hydrolyzate solution. Prepared as described under "Nicotinic Acid Assay," p. 364, 10% solution.

These stock solutions can be preserved by refrigeration or by intermittent steam sterilization for considerable periods of time. To prepare 100 cc. of the basal medium (sufficient for 20 assay tubes) the stock solutions are mixed in the following ratios: sugar and salts solution, 50 cc.; potassium citrate buffer, 10 cc.; ammonium sulfate solution 5 cc.; inositol solution, 5 cc.; calcium pantothenate solution, 5 cc.; pyridoxine solution, 5 cc.; thiamine solution, 5 cc.; casein hydrolyzate solution, 10 cc.; and water to 100 cc. The mixed medium is stable for several weeks if kept a few degrees below zero.

Procedure—Five cc. of the basal medium plus a solution of the unknown or an aliquot of the standard biotin solution are placed in a series of test tubes (1 × 8 in.) with sufficient water to make the total volume 9.0 cc. in each case. Suitable amounts of biotin to establish a standard curve are 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 mγ of biotin, and extracts are assayed at levels calculated to furnish several assay points within this range. The tubes are plugged with cotton, steamed for 10 min., cooled, and inoculated with 1 cc. of the inoculum suspension. The tubes are then shaken mechanically for 16-18 hr. at 30° C. The growth in each tube is then determined in the photoelectric colorimeter, the standard curve plotted, and the results calculated in the usual fashion.

Comments—No extensive results obtained by this procedure have been published. The procedure is very similar to procedures for pyridoxine (221) and pantothenic acid (30), which have been published and which give very excellent results. The medium permits growth which is superior to that obtained on either the medium of Snell *et al.* or of Hertz (Media 32 and 33, Table XIII).

B. PANTOTHENIC ACID

(1) Assay Media and Their Preparation

Considering that pantothenic acid was originally discovered by its effect on yeast growth, and that this organism played a predominant role in work leading to characterization and synthesis of the vitamin (144), it is strange that more yeast growth methods for the determination of this substance in natural materials have not been developed. In Table XIV are presented two assay media for this purpose. Medium 35 of Williams and Saunders (283) is given for its historical importance only; it was with media similar to, or identical with this that the early work resulting in discovery and elucidation of the structure of pantothenic acid was done. Although suitable for measuring the pantothenic acid content of concentrates of this substance, if used with the proper strain of yeast, it is not suitable for use with natural materials (47, 29), and the method will not be considered further. The more recent method of Atkin *et al.* (30), on the other hand, is quite satisfactory. For those equipped for and accustomed to using yeast as an assay organism, the method is simpler and faster than those which use lactic acid bacteria for the determination of this vitamin.

(2) The Assay Methods

a. Method of Atkin, Williams, Schultz and Frey (30).

Assay organism and inoculum—*Saccharomyces carlsbergensis* 4228, ATCC No. 9080. Carried as slant cultures on Difco malt agar. Transferred at monthly intervals; incubated 24 hr. at 30° C. following transfer, then held in the refrigerator. The day before an assay run, a fresh transfer is made and incubated for 24 hr. Yeast is transferred from this slant to a tube of sterile saline until the light absorption indicates that the concentration is 3 mg/cc. The calibration is based on a known suspension of moist baker's yeast. A 10 cc. aliquot of this suspension is added to 90 cc. of sterile saline contained in an Erlenmyer flask. This final suspension contains 0.3 mg. of moist yeast/cc. and is ready for use.

Basal medium—Medium 36, Table XIV. The medium is prepared by mixing a series of stock solutions, which contain the following ingredients:

(1) Sugar and salts solution. One liter contains 200 g. of C.P. dextrose, 2.2 g. KH_2PO_4, 1.7 g. KCl, 0.5 g. $MgSO_4.7H_2O$, 0.5 g. $CaCl_2.2H_2O$, 0.01 g. $MnSO_4$, and 0.01 g. $FeCl_3$.

(2) Potassium citrate buffer. One liter contains 100 g. of potassium citrate monohydrate and 20 g. of citric acid monohydrate.

(3) Thiamine solution. 10 γ/cc.
(4) Pyridoxine hydrochloride solution. 10 γ/cc.
(5) Inositol solution. 1 mg./cc.
(6) Biotin solution. 0.8γ/cc.
(7) Ammonium sulfate solution. 150 mg./cc.
(8) Asparagine solution. 30 mg./cc. (heat to dissolve).

The solutions are sterilized by heating in flowing steam for 30 min. on 3 successive days, and may be stored at room temperature (but protect pyridoxine solution from light!) until used. To prepare 100 cc. of the basal medium (sufficient for 20 assay tubes) from the stock solutions

TABLE XIV

Composition of Assay Media for Pantothenic Acid

	Medium No.	
Constituent	35. Williams and Saunders, 1934 (283) (*Sacch. cerevisiae* G.M.)	36. Atkin, Williams, Schultz and Frey, (30) (*Sacch. carlsbergensis* 4228)
	Amount/100 cc. of basal medium[a]	
Glucose	—	10 g.
Sucrose	2.0 g.	—
Asparagine	150 mg.	375 mg.
Biotin	—	4 γ
Inositol	—	5 mg.
Pyridoxine hydrochloride	—	50 γ
Thiamine chloride	—	50 γ
Ammonium sulfate	300 mg.	750 mg.
Potassium citrate (monohydrate)	—	1.0 g.
Citric acid (monohydrate)	—	200 mg.
KH_2PO_4	200 mg.	110 mg.
KCl	—	85 mg.
$CaCl_2.2H_2O$	25 mg.	25 mg.
$MgSO_4.7H_2O$	25 mg.	25 mg.
H_3BO_3	100 γ	—
$ZnSO_4$	100 γ	—
$MnCl_2$	100 γ	500 γ[b]
$TlCl_3$	100 γ	—
$FeCl_3$	50 γ	500 γ
$CuSO_4.5H_2O$	10 γ	—
KI	10 γ	—

[a] Medium 35 is 1.2 times its final concentration; medium 36 is twice its final concentration.

[b] $MnSO_4$ used in place of $MnCl_2$.

these are mixed as follows: sugar and salts solution, 50 cc.; potassium citrate buffer, 10 cc.; inositol solution, 5 cc.; ammonium sulfate solution, 5 cc.; thiamine solution, 5 cc.; pyridoxine solution, 5 cc.; biotin solution, 5 cc.; asparagine solution, 12.5 cc.; and water to 100 cc. The medium need not be prepared fresh for each run; large batches may be mixed and stored at temperatures a few degrees below zero for as long as 3 months with no observed ill effects upon the assay.

Procedure—Five cc. of the basal medium plus a solution of the unknown or an aliquot of the pantothenate standard solution are placed in a series of test tubes (25 × 250 mm.) with sufficient water to make the total volume 9.0 cc. in each case. Suitable amounts of calcium pantothenate to establish a standard curve are 0, 50, 100, 150, 200, 300, and 400 mγ, and extracts are assayed in amounts considered by approximation to furnish pantothenate within this concentration range. The tubes are plugged with cotton, steamed for 10 min., cooled, and inoculated with 1 cc. of the inoculum suspension. The tubes are then shaken mechanically at 30° C. for 16-18 hr. The growth in each tube is then estimated turbidimetrically in the photoelectric colorimeter, the standard curve constructed, and the results calculated.

Comment—One of the reasons yeast growth methods were not earlier adapted for pantothenic acid assay in natural materials was the fact that β-alanine exerts the same stimulating effect as does pantothenic acid on yeast growth. It was early recognized that the effectiveness of β-alanine in this respect was greatly enhanced if asparagine was eliminated from the medium, and could be suppressed to a large extent by addition of asparagine (262, 283). In the above method, the effect of β-alanine has been completely eliminated, for all practical purposes, by inclusion of a high level of asparagine in the medium. The specificity of the assay method is well supported by data included in the original paper (30). Drifts are insignificant, and recoveries of added pantothenic acid varied between 97 and 116%, with an average of 103.6%. Reproducibility of the method is excellent: 10 separate assays of a refrigerated sample of dried yeast gave a mean value of 166.3 γ of pantothenate/g. with an average deviation of 3.2% and a standard deviation of 3.8%.

C. VITAMIN B_6

(1) Assay Media and Their Preparation

The first method and medium presented for assay of vitamin B_6 with yeast was that of Williams *et al.* (284), and those of Siegal *et al.* (224) and Burkholder (268) are modifications of this early medium. The composition of the various assay media is given in Table XV.

Special supplements for Media 37 and 39 are prepared as follows (284).

a. Vitamin B_6-Free Liver Extract. Five g. of liver powder or paste (Wilson Laboratories 1-20) are mixed with 195 cc. of water and autoclaved at 15 lb. pressure for 15 min. After cooling, the pH is adjusted with HCl to 1.0, and 10 g. of fuller's earth (Braun Corp.) is added. The mixture is shaken several times daily for 4 days. The supernatant liquid is then decanted, adjusted to pH 4.5-5.0, and pipetted in 5 cc. lots into a series of test tubes. These are plugged with cotton and autoclaved 15 min. at 15 lb. Three days later, the autoclaving is repeated. The tubes are then stored until use. Two cc. of this solution/100 cc. of medium supplies the proper amount of the supplement.

b. Vitamin B_6-Free Yeast Extract. Five g. of Difco yeast extract are treated in the same way as the liver extract above. Two cc. of the resulting solution added to 100 cc. of medium supplied the proper amount of the supplement.

Siegel *et al.* (224) found that, in their hands, the above procedures failed to remove vitamin B_6 quantitatively from these extracts;[45] they modified the preparation for use with medium 38 in the following manner (224).

c. Vitamin B_6-Free Liver and Yeast Extract Solutions. To the acidified (pH 1.0) liver and yeast extract solutions described by the original authors (see above) are added 2 g. of Lloyd's reagent and the suspension is shaken occasionally during the next 24 hr. After filtration, the solution is treated again with the same quantity of adsorbent. The mixture is then centrifuged, and the clear supernatant removed, adjusted to pH 4.5-5.0, bottled, and sterilized in the autoclave for 15 min. at 15 lb. pressure. Two cc. of each solution, thus prepared, supplies the proper amount of supplement for 100 cc. of the basal medium.

A suitable casein hydrolyzate for use with all media can be prepared with HCl as described on p. 364. Rabinowitz and Snell (219), using the method of Atkin *et al.* with medium 40, found a hydrolyzate prepared as described on p. 411 to give less growth in tubes not supplemented with vitamin B_6.

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[45] The original authors noted occasional difficulties of the same nature. A suitable supplement could be obtained only with a special type of fuller's earth obtained from the Braun Corporation.

TABLE XV

Composition of Assay Media for Vitamin B_6

Constituent	Medium No.			
	37. Williams, Eakin and McMahan, 1941 (284) (*Sacc. cerevisiae*, G.M.)	38. Siegel, Melnick and Oser, 1943 (224) (*Sacc. cerevisiae*, G.M.)	39. Burkholder 1943 (268) (*Sacc. oviformis*, 782)	**40. Atkin, Schultz, Williams and Frey, 1943 (221)** (*Sacc. carlsbergensis* 4228)
	Amount/100 cc. of basal medium			
Glucose	—	—	2.0 g.	10 g.
Sucrose	2.0 g.	2.0 g.	—	—
$(NH_4)_2SO_4$	300 mg.	300 mg.	200 mg.	—
KH_2PO_4	200 mg.	200 mg.	150 mg.	110 mg.
KCl	—	—	—	85 mg.
$CaCl_2.2H_2O$	25 mg.	25 mg.	33 mg.	25 mg.
$MgSO_4.7H_2O$	25 mg.	25 mg.	50 mg.	25 mg.
H_3BO_3, $ZnSO_4$, $MnCl_2$, $TlCl_3$	100 γ of each	100 γ of each	[a]	—
$MnSO_4$	—	—	[a]	500 γ
$FeCl_3$	50 γ	50 γ	[a]	500 γ
$CuSO_4.5H_2O$	10 γ	10 γ	[a]	—
KI	10 γ	10 γ	10 γ	—
L-Asparagine	300 mg.	300 mg.	200 mg.	—
L-Tryptophan	—	200 γ	—	—
Hydrolyzed casein, charcoal-treated	20 mg.	250 mg.	50 mg.	800 mg.

TABLE XV (continued)

Constituent	Medium No. 37. Williams, Eakin and McMahan, 1941 (284) (*Sacc. cerevisiae*)	38. Siegel, Melnick and Oser, 1943 (224) (*Sacc. cerevisiae*)	39. Burkholder 1943 (268) (*Sacc. oviformis*, 782)	40. Atkin, Schultz, Williams and Frey, 1943 (221) (*Sacc. carlsbergensis* 4228)
	Amount/100 cc. of basal medium			
Liver extract, vitamin B_6-free	50 mg.-equiv.	50 mg.-equiv.	50 mg.-equiv.	—
Yeast " " "	50 mg.-equiv.	50 mg.-equiv.	50 mg.-equiv.	—
Biotin	0.02 γ	0.1 γ	0.2 γ	1.6 γ
Calcium pantothenate	30 γ	30 γ	20 γ	500 γ
Inositol	500 γ	500 γ	—	5000 γ
Thiamine	2 γ	2 γ	20 γ	50 γ
Folic acid	0.05 γ	0.05 γ	—	—
Potassium citrate, monohydrate	—	—	—	1 g.
Citric acid, monohydrate	—	—	—	200 mg.

[a] Trace elements were added in parts per million as follows: B, 0.01; Mn, 0.01; Zn, 0.07; Cu, 0.01; Mo, 0.01; and Fe, 0.05.

(2) The Assay Methods

a. Method of Williams, Eakin and McMahan (284).

Assay organism—*Saccharomyces cerevisiae* G.M.[46] ATCC No. 7752. Carried by weekly transfer on molasses-agar slants.

Basal medium—Medium 37, Table XV.

Sterilization—All medium and equipment to be sterilized is heated for 10 min. in live steam at 100° C.

Inoculum—Yeast removed from a slant stock culture is suspended in a small volume of the previously sterilized basal medium, and its concentration determined. Enough of this heavy suspension is added to the previously sterilized basal medium to furnish 0.05 mg. of moist yeast/100 cc. of basal medium.

Procedure—Assays are carried out in test tubes supported in a wire rack. Standard pyridoxine solutions and unknown samples are added in volumes of 0.01-0.1 cc. The assay range is from 0-0.4 mγ of pyridoxine hydrochloride. Standard and samples are sterilized, cooled, and 2 cc. of the inoculated basal medium added to each tube.

Incubation—Assays are incubated in a constant temperature bath without stoppers and without agitation for 15 hr. at 30° C.

Measurement of response—Assay tubes are diluted with 5 cc. of saturated aqueous chlorothymol solution, shaken well, and the culture turbidity determined with the photoelectric colorimeter.

Comment—The growth differential obtained between tubes supplemented and unsupplemented with vitamin B_6 is not sufficient for accurate determination with most photoelectric colorimeters. The special instrument described by Williams *et al.* (28) is suitable.

In its original application, the method gave extremely low results for the vitamin B_6 content of samples, as compared with those determined by other methods. In part, this was undoubtedly due to failure to use a suitable extraction procedure for vitamin B_6. It is now known, too, that under these testing conditions, pyridoxal and pyridoxamine have only 0.2-0.6 of the activity of pyridoxine for the assay organism used (225).

b. Method of Siegel, Melnick and Oser (224).

Assay organism—*Saccharomyces cerevisiae* G.M. ATCC No. 7752. Carried by daily transfer on Difco malt agar slants, incubated at 30° C.

Basal medium—Medium 38, Table XV.

Sterilization and procedure—These follow those of the original method, described above, in all details. The modified medium permits much heavier growth of yeast, so that the assay range has been shifted upwards to 0-4.0 mγ of pyridoxine/2 cc. Standard and samples are tested at 10 assay levels arithmetically spaced between these limits.

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[46] G.M. = Gebrüder Mayer.

Incubation—Without agitation in the water bath at 31° C. for 16-18 hr.

Measurement of response—Turbidimetric, after dilution of each culture with 5 cc. of aqueous, saturated chlorothymol solution.

Comment—Growth of the test organism is much heavier on this modified medium than on the original medium and is readily determined with any photoelectric colorimeter; assay values obtained are in fair agreement with others in the literature. Pyridoxamine and pyridoxal, under these testing conditions, are only 40-46% as active as pyridoxine; substances containing most of their vitamin B_6 in these forms might be expected, therefore, to give low values by this method if a pyridoxine standard were used. However, when fed in the ration, pyridoxal and pyridoxamine are also less active than pyridoxine for animals (286); it is possible, therefore, that a method such as this one would reflect more accurately the nutritionally available vitamin B_6 for animals than one (such as that of Atkin *et al.* (221)) in which the three forms of vitamin B_6 are equally active. Such questions might be further investigated, and the method of assay intelligently chosen to fit the purpose of the investigation.

c. *Method of Burkholder (268).*

Assay organism—*Saccharomyces oviformis* 782. Carried on yeast-peptone-dextrose agar slants. Frequency of transfer or exact formula of medium not specified.

Basal medium—Medium 39, Table XV.

Sterilization—Steaming for 10 min. in the autoclave at atmospheric pressure.

Inoculum—Yeast removed from a slant culture is suspended in a small volume of previously sterilized basal medium, and its concentration determined. By calculation enough of this suspension was transferred to the larger volume of basal medium to supply 0.2 mg. of moist yeast /100 cc. of solution.

Procedure—Assays are carried out in standard culture tubes. Standard and sample solutions in graded amounts not over 1 cc. are added to these tubes, and the volume adjusted in each case to 1 cc. with distilled water. The assay range is from 0 to 8 mγ of pyridoxine. All tubes are sterilized by steaming; after cooling, 5 cc. of the inoculated basal medium are added to each tube.

Incubation—Tubes are incubated without plugs or shaking in the water bath at 30° C. for 20 hr.

Measurement of response—Turbidimetric.

Comment—The method has not been applied to natural materials. Pyridoxal and pyridoxamine are from 0.2 to 0.8 as active as pyridoxine for this organism, depending upon the cultural conditions and time of incubation (225). In this respect, therefore, the organism resembles *Saccharomyces cerevisiae* G.M. used in the two previously considered methods.

d. Method of Atkin, Schultz, Williams and Frey (221).

Assay organism—*Saccharomyces carlsbergensis* 4228, ATCC No. 9080. Carried on Difco malt agar slants with transfer at biweekly intervals. Incubated at 30° C. for 24 hr., then held in refrigerator between transfers.

Basal medium—Medium 40, Table XV.

Sterilization—By steaming for 10 min. at atmospheric pressure.

Inoculum—24 hr. preceding an assay, a fresh transfer of the test organism is made and incubated at 30° C. When the assay is ready for inoculation yeast from this culture is transferred by sterile wire loop to 10 cc. of sterile saline in a colorimeter tube. The density of the suspension is adjusted so that it contains 1 mg. of yeast/cc. 5 cc. of this suspension are then added to 45 cc. of saline in an Erlenmeyer flask. One cc. of this suspension is used to inoculate each assay tube.

Procedure—The entire procedure is exactly analogous to those of the same authors described in detail under "Biotin" and "Pantothenic acid"; the same solutions may be used in mixing the basal medium. Assays are carried out in 1 × 8″ culture tubes. Standard pyridoxine solutions and unknown samples are added in volumes ranging from 0 to 4 cc. 0, 5, 10, 15, 20, 30, and 40 mγ of pyridoxine hydrochloride are suitable concentrations to establish the standard curve. The volume of each tube is adjusted to 4 cc. with distilled water, then 5 cc. of the basal medium are added. All tubes are plugged, sterilized, cooled and inoculated with 1 cc. of the yeast inoculum.

Incubation—Tubes are shaken at 30° C. for 16-18 hr.

Measurement of response—Turbidimetric.

Comment—Since its introduction this method has been widely used for determination of vitamin B_6 with uniformly satisfactory results. It is also the simplest of the methods so far devised. Growth in the medium is heavy, and the yeast responds equally to pyridoxine, pyridoxal and pyridoxamine (225).[47] It can, therefore, be used in conjunction with assays with lactic acid bacteria to determine the distribution of these forms of vitamin B_6 in natural materials (234, 236). Several minor procedural variations have been suggested (*e.g.*, 234, 219) but these apparently have served chiefly to adapt the method to special equipment or practices of the operators, and have not improved the specificity of the assay as originally suggested. An exception to this latter statement was discovered by Hopkins and Pennington (286), who found it advisable to add nicotinic acid to the basal medium at a level of 0.5 mg./100 cc. to prevent obtention of occasional high assay values. The value of this suggestion has been confirmed (236). It seems worth while to point out that the assay can be carried out successfully without shaking, with only a slight decrease in uniformity of assay results. When this is done, the tubes should be incubated at an angle of 25° or less from the horizontal, to increase the ratio of surface to volume.

Rabinowitz and Snell have used this method in conjunction with assays for vitamin B_6 which employ *Streptococcus faecalis* (p. 413) and *Lactobacillus casei* (p. 411) to permit the differential determination of pyridoxal, pyridoxamine and pyridoxine in natural materials (236). They find that almost all of the vitamin B_6 of animal tissues and yeast is present as pyridoxal and pyridoxamine; little or no pyridoxine can be detected. Plant tissues contain all three forms of the vitamin.

D. INOSITOL

(1) Extraction from Natural Materials

The biological activity of inositol was first established by Eastcott (3) with yeast. Woolley later showed that it was also required in the diet of the mouse (287, review: 288), a finding which has greatly stimulated the interest in this substance. Inositol occurs free in some tissues and also combined as phytin, as a component of some phosphatides, and perhaps in other forms (288). These combined forms are essentially inactive in promoting yeast growth, but have full activity for the animal (288, 289). It is, therefore, necessary to hydrolyze tissues before analysis for their total inositol content.

Inositol is very stable to acid hydrolysis. The following procedure (290) is the most effective extraction procedure of those so far suggested, and has been most commonly followed (290, 282, 291).

A suitably sized sample is refluxed with 18% HCl for 6 hr. The HCl is removed by distillation *in vacuo* and the residue taken up in water, adjusted to the pH of the assay medium being used, and an aliquot suitably diluted for assay. Direct neutralization of the HCl cannot be used because of toxicity of large amounts of salt for the test organisms. The volume of HCl used for the hydrolysis is not important, as long as an excess is present. A volume of 200 cc. for a sample containing 0.3-0.4 mg. of inositol has been recommended (282).

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[47] Occasionally, pyridoxamine shows somewhat less activity, 0.7-0.9 that of pyridoxine or pyridoxal (cf. 236).

The amount of inositol extracted from tissues is also increased by enzymatic digestion (78); however, for most tissues, this procedure is less effective than acid hydrolysis.

(2) Assay Media and Their Preparation

Although inositol was the first constituent of "bios" to be identified, suitable media for its quantitative determination in natural materials were developed only following identification of other vitamins required by yeast. Presence of these substances greatly increases the magnitude of responses to inositol. The composition of suggested media for this purpose is given in Table XVI. The simplest of these to prepare and the most satisfactory in practical use is that of Atkin *et al.* (Medium 43).

a. Preparation of medium No. 41 (Woolley (290)). Vitamin free-casein is hydrolyzed by heating in an autoclave at 15 lb. pressure for 16 hr. with 7 *N* H_2SO_4. The latter is removed from the hydrolyzate with $Ba(OH)_2$. The filtrate is adjusted to pH 6.0. An aliquot of this hydrolyzate equivalent to 2.5 g. of casein is mixed with 100 g. of glucose, 8.3 g. of NH_4NO_3, 4.2 g. of KH_2PO_4, 2.1 g. of $MgSO_4.7H_2O$, 0.7 g. of $CaCl_2.2H_2O$, and approximately 300 cc. of H_2O, and heated in an autoclave at 15 lb. pressure for 15 min. The precipitate which forms is removed, and to the filtrate is added the non-dialyzable portion of 10 g. of rice bran extract (Vitab, obtained from National Oil Products Company). This is prepared by dissolving the rice bran extract in water, transferring to viscose sausage casing, and dialyzing against running water for 48 hr. The mixture of vitamins and growth factors (0.5 mg. thiamine, 0.5 mg. riboflavin, 0.5 mg. pyridoxine, 1 mg. nicotinic acid, 2.5 mg. choline chloride, 1 mg. pimelic acid, 5 mg. asparagine, 0.05 mg. biotin, 0.5 mg. of calcium pantothenate, 2.5 mg. uracil and 2.5 mg. adenine) is now added, and, finally, the bios II concentrate of Lucas (292) in an amount equivalent to 10 g. of malt sprouts. The volume is now adjusted to 500 cc. and preserved under toluene until used.

b. Preparation of Unusual Ingredients of Medium No. 42 (Williams et al. (293)). The only unusual feature of this medium is the inclusion of a calf liver autolyzate, which is stated to supply missing stimulative factors without supplying interferingly large amounts of inositol. This is prepared as follows. A weighed portion of calf's liver is homogenized in the Potter-Elvehjem apparatus (294), covered with benzene, and placed in an incubator at 37° C. for 24 hr. The contents are then washed into a beaker, made approximately 0.1 *N* with acetic acid, and steamed in an autoclave for 30 min. After filtration, the extract is diluted so that 1 cc. is equivalent to 75 mg. of original tissue. The extract is dispensed to a number of smaller vessels, which are plugged with cotton and sterilized. The casein hydrolyzate required for the medium may be prepared as described on p. 364.

c. Preparation of Hydrolyzed Casein for Medium No. 43 (Atkin et al. (282)). The casein hydrolyzate is prepared according to the standardized procedure described on p. 364.

(3) The Assay Methods

a. Method of Woolley (290, 290a).

Assay organism—*Saccharomyces cerevisiae,* Hansen No. 1. Carried as slants on the basal medium supplemented with the aqueous extract of 10 g. of malt sprouts/100 cc. and solidified with 1.5-2.0% agar.

Basal medium—Medium 41, Table XVI, prepared as described in section 2, immediately above.

Sterilization—By autoclaving at 15 lb. pressure for 15 min.

Inoculum—Organisms transferred from a slant to liquid medium consisting of the basal medium supplemented with aqueous extract of 10 g. of malt sprouts/100 cc. Incubated 24 hr. at 30° C., cells collected by centrifugation and washed 3 times with sterile phosphate buffer (presumably about 0.1%, pH 5.0-6.0; not specified), then suspended in a volume of buffer 20 times that of the original culture. One drop (0.05 cc.) of this suspension is used to inoculate each assay flask; such an inoculum contains about 70,000 cells.

Procedure—Five cc. portions of the basal medium are placed in 50 cc. Erlenmeyer flasks. Aliquots of the samples and of a standard inositol solution are added to the various flasks in volumes not exceeding 6 cc. The assay range is from 0 to 20 γ of inositol per flask. All volumes are adjusted to 11 cc., and the flasks plugged, sterilized, cooled and inoculated with 1 drop of the inoculum suspension.

Incubation—Incubated in a water bath held at 30° C. for a 16 hr. period.

Measurement of response—Turbidimetric.

Comment—When the basal medium is properly prepared, growth of yeast is very slight in the absence of inositol and heavy in its presence, and good assay results are secured. In the light of present knowledge, however, the basal medium is needlessly complex and difficult to prepare. The procedure of Atkin *et al.* (282) with medium 43 will be found just

TABLE XVI

Composition of Assay Media for Inositol

Constituent	41. Woolley, 1941 (290) (*Sacc. cerevisiae*)	42. Williams, Stout, Mitchell and Mc-Mahan 1941 (293) (*Sacc. cerevisiae* G.M.)	43. Atkin, Williams, Schultz and Frey, 1944 (282) (*Sacc. carlsbergensis* 4228)
		Amount/100 cc. of basal medium[a]	
Glucose	20 g.	—	10 g.
Sucrose	—	2.0 g.	—
$(NH_4)_2SO_4$	—	300 mg.	750 mg.
NH_4NO_3	1.66 g.	—	—
KH_2PO_4	840 mg.	200 mg.	110 mg.
KCl	—	—	85 mg.
$MgSO_4.7H_2O$	420 mg.	25 mg.	25 mg.
$CaCl_2.2H_2O$	140 mg.	25 mg.	25 mg.
$FeCl_3$	—	50 γ	500 γ
$MnSO_4$	—	—	500 γ
H_3BO_3, $ZnSO_4$, $MnCl_2$, $TlCl_3$	—	100 γ of each	—
$CuSO_4$, KI	—	10 γ of each	—
Potassium citrate, monohydrate	—	—	1 g.
Citric acid, monohydrate	—	—	200 mg.
L-Asparagine	1 mg.	300 mg.	—
Hydrolyzed casein	500 mg.	20 mg.	1 gm.
Biotin	10 γ	0.02 γ	5 γ
Calcium pantothenate	100 γ	30 γ	500 γ
Pyridoxine hydrochloride	100 γ	2 γ	50 γ
Thiamine chloride	100 γ	2 γ	50 γ
Choline chloride	500 γ	—	—
Folic acid	—	0.05 γ	—

TABLE XVI (continued)

Constituent	41. Woolley, 1941 (290) (*Sacc. cerevisiae*)	42. Williams, Stout, Mitchell and McMahan 1941 (293) (*Sacc. cerevisiae* G.M.)	43. Atkin, Williams, Schultz and Frey, 1944 (282) (*Sacc. carlsbergensis* 4228)
		Amount/100 cc. of basal medium[a]	
Nicotinic acid	200 γ	—	—
Pimelic acid	200 γ	—	—
Riboflavin	100 γ	—	—
Adenine	500 γ	—	—
Uracil	500 γ	—	—
Calf liver autolyzate	—	—	—
Dialyzed vitab	2 g.-equiv.	75 mg.-equiv.	—
Bios II concentrate	2 g.-equiv.	—	—

[a] Media 41 and 43 are twice their final concentration; medium 42 is 1.2 times its final concentration.

as accurate and far simpler in application. Jurist and Foy (291) have modified the medium of Woolley by eliminating the two troublesome components, the dialyzed vitab preparation and the bios II concentrate, but details of their modification are not available.

The only thorough study of the biological specificity of inositol in promoting growth of yeast was made with this method (289). Combined forms of *meso*-inositol, such as phytin, soybean cephalin, *etc.*, were inactive for the yeast. Related compounds which occur naturally, and all of which had negligible activity (< 1%) compared to *meso*-inositol were: *l*-inositol, *d*-inositol, quercitol, quebrachitol, pinitol, quinic acid, and inosose. Mytilitol (a methyl inositol) was about 10% as active as inositol for yeast. Where tested, activity of these compounds for mice corresponded to their activity for yeast.

Presumably, but not certainly, the specificities shown by this organism would hold also for other strains of yeast.

b. Method of Williams, Stout, Mitchell and McMahan (293).

Assay organism—*Saccharomyces cerevisiae* G.M.; ATCC No. 7752. Carried by weekly transfer on molasses agar-slants.

Basal medium—Medium 42, Table XVI. For preparation of unusual ingredients of medium, see section 2 above.

Sterilization—By steaming for 10 min. at atmospheric pressure.

Inoculum—Yeast from a slant culture is suspended uniformly in a small amount of medium, and its concentration determined turbidimetrically by use of an appropriate calibration curve. By calculation, enough of this suspension is added to the previously sterilized basal medium to bring the final concentration to 0.15 mg. of moist yeast/100 cc.

Procedure—Assays are carried out in test tubes supported in a wire rack. Standard inositol solutions and unknown samples are added in volumes of 0.01 to 0.1 cc. The assay range is from 0 to 1 γ of inositol. Standard and samples are sterilized, cooled, and 2 cc. of the inoculated basal medium are added to each tube.

Incubation—Assay tubes are incubated at 30° C. in a constant temperature bath for a period of 15 hr.

Measurement of response—Contents of each tube are diluted with 5 cc. of aqueous saturated chlorothymol solution, shaken well, and the culture turbidity determined in a photoelectric colorimeter or other suitable measuring instrument (28).

Comment—The magnitude of the growth differential between supplemented and unsupplemented assay tubes is not sufficient for accurate determination in most photoelectric colorimeters, but is readily determined in the turbidimeter of Williams *et al.* (28). Additional disadvantages of the method are: (1) The calf's liver supplement may sometimes contain interferingly large amounts of inositol (considered by the authors of the method to be any amount over 500 γ/g. of liver) and (2) extracts of many materials contain sufficient amounts of inhibitory or stimulatory substances to interfere markedly with accurate assay. In the latter cases, the authors recommend replacing the autolyzed liver supplement of the medium with an autolyzate of the substance to be assayed. The amount of the latter which can be used depends upon its inositol content. An amount which furnishes not more than 0.5 γ of inositol/cc. of basal medium is recommended.

c. Method of Atkin, Williams, Schultz and Frey (282).

Assay organism—*Saccharomyces carlsbergensis* 4228; ATCC No. 9080. Carried on Difco malt agar slants with transfers at biweekly intervals. Incubation at 30° C. for 24 hr., then held in refrigerator between transfers.

Basal medium—Medium 43, Table XVI.

Sterilization—By steaming for 10 min. at atmospheric pressure.

Inoculum—24 hr. before an assay, a fresh slant of the test organism is prepared and incubated at 30° C. When the assay is ready for inoculation, yeast from this culture is transferred with a sterile wire loop to 10 cc. of sterile saline. The density of this suspension is adjusted so that it contains 1 mg. of yeast/cc. Five cc. of this suspension are then added to 45 cc. of saline in an Erlenmeyer flask. One cc. of this suspension is used to inoculate each assay tube.

Procedure—The procedure is analogous to those of the same authors for biotin, pantothenic acid and vitamin B_6, the former two of which were described in detail. The same solutions (inositol omitted) may be used in mixing the basal medium. Assays are performed in $1 \times 8''$ test tubes. Standard inositol solutions and unknown samples are added in volumes ranging from 0 to 4 cc. 0, 1, 2, 4, 6, 8 and 10 γ of inositol are suitable concentrations to establish the standard curve. The volume in each tube is adjusted to 4 cc. with distilled water, then 5 cc. of the basal medium are added. All tubes are plugged, sterilized, cooled, and inoculated with 1 cc. of yeast inoculum.

Incubation—Tubes are shaken mechanically at 30° C. for 16-18 hr.

Measurement of response—Turbidimetric.

Comment—Of the three suggested methods for inositol, this is the simplest, and generally the most satisfactory.

E. NICOTINIC ACID

(1) Assay Media and Their Preparation

Although a large number of yeasts are now known which require nicotinic acid for growth, few have been suggested as assay organisms, since the microbiological method of Snell and Wright and its modifications, which utilize *Lactobacillus arabinosus* as test organism, have proved very satisfactory. Very recently, however, Williams (295) has developed a method using the yeast, *Torula cremoris,* which is useful for this purpose. Like other yeast methods, the recommended incubation period is only 16 hr., as compared with 40-72 hr. for the methods employing lactic acid bacteria. The organism differs somewhat from *L. arabinosus* in specificity of response to naturally-occurring compounds related to nicotinic acid, and can, therefore, be applied for additional purposes (see below).

The composition of the assay medium used is shown in Table XVII. The general techniques used in the assay are very similar to those used by Atkin *et al.* in their methods for biotin, pantothenic acid, vitamin B_6, and inositol, all of which have been described above.

The charcoal-treated peptone for the medium is prepared as follows (296): 100 g. of Bacto peptone (Difco Laboratories, Detroit) are dissolved in 800 cc. of distilled water. The pH of the solution is adjusted to 3.0 with concentrated HCl. A faint cloud appears at this point. Twenty g. of activated charcoal, Darco G-60, are added and the mixture stirred mechanically for 1 hr. The solution is filtered by suction, the pH readjusted to 3.0, and the charcoal treatment repeated, but with only 10 g. of charcoal. The filtrate should have no more than a faint tinge of color. The volume is adjusted to 1 l. (10% on basis of original peptone) and the solution preserved under toluene.

The ingredients of the medium can be combined conveniently by preparing three solutions, (a) charcoal-treated peptone as above, (b) potassium citrate buffer (100 g. potassium citrate, 20 g. citric acid/1) and (c) vitamins, biotin 1 γ, pyridoxine 100 γ, thiamine 100 γ, calcium pantothenate 100 γ, and inositol 1 mg./cc. To prepare medium for 20 tubes (100 cc. of basal medium), 20 cc. of (a), 10 cc. of (b) and 5 cc. of (c) are combined with 10 g. of glucose, 60 mg. KH_2PO_4 and 20 mg. of $MgSO_4.7H_2O$, and the mixture diluted to 100 cc.

(2) The Assay Methods

a. Method of Williams (295).

Assay organism—*Torula cremoris,* ATCC No. 2512. Carried on Difco malt agar slants with monthly transfer. Incubated at 37° C. for 24 hr. following transfer, then held in refrigerator.

Basal medium—Medium 44, Table XVII.

TABLE XVII

Composition of Assay Medium for Nicotinic Acid

Medium No. 44. Williams, 1946 (295)

Constituent	Amount/100 cc. of basal medium[a]	Constituent	Amount/100 cc. of basal medium[a]
Glucose	10 g.	Biotin	5 γ
KH_2PO_4	60 mg.	Thiamine hydrochloride	500 γ
$MgSO_4.7H_2O$	20 mg.	Pyridoxine hydrochloride	500 γ
Potassium citrate monohydrate	1.0 g.	Calcium pantothenate	500 γ
Citric acid, monohydrate	200 mg.	Inositol	5 mg.
Peptone, charcoal-treated	2.0 g.		

[a] The medium is twice its final concentration.

Sterilization—By heating in flowing steam at atmospheric pressure for 10 min.

Inoculum—24 hr. before an assay run, a fresh transfer of yeast is prepared and incubated at 37° C. Immediately before use, yeast from the slant is removed with a sterile wire loop to 10 cc. of sterile saline in an Evelyn tube until it reads 15-25% absorption (galvanometer reading in Evelyn colorimeter: 75-85). The contents of this tube are then transferred to 90 cc. of sterile saline. One cc. of this diluted inoculum is used for each assay tube.

Procedure—Standard Evelyn colorimeter tubes are used for assay.[48] Five cc. of basal medium is added to each tube. Standard nicotinic acid solutions and unknown samples are added in volumes ranging from 1 to 4 cc. 0, 25, 50, 75, 100, 125, 150, and 200 mγ of nicotinic acid are suitable concentrations to establish the standard curve; four points within this range should be obtained for each sample. All tubes are diluted to 9 cc. with water, plugged, sterilized, cooled, and inoculated with 1 cc. of inoculum suspension.

Incubation—Tubes are shaken mechanically for 16-18 hr. at any temperature between 30 and 38° C. Satisfactory results are obtainable as early as 12 or as late as 40 hr.

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[48] **Alternatively, ordinary 1 × 8" test tubes can be used, and their contents transferred to an Evelyn colorimeter tube for final evaluation of the turbidity.**

Measurement of response—Turbidimetric.

Comment—Nicotinamide and nicotinic acid are equally active for the test organism. In contrast to *L. arabinosus*, for which trigonelline is inactive, this compound is 85% as active as nicotinic acid for *T. cremoris*, the test organism used. N′-methylnicotinamide is inactive, but is rendered active by hydrolysis with 1 *N* acid or 1 *N* alkali at 120° C. for 30 min., presumably by conversion to trigonelline. Treatment with stronger alkali completely inactivates both trigonelline and N′-methylnicotinamide for the test organism. By appropriate treatment of the sample one may estimate, therefore, (1) niacin, (2) niacin plus trigonelline, and (3) niacin plus trigonelline plus N′-methylnicotinamide. The original paper (295) must be consulted for details. For specific estimation of nicotinic acid (and substances such as nicotinamide and the coenzymes hydrolyzable to it), the samples are hydrolyzed with 3 *N* NaOH at 120° C. for 1 hr. Under these conditions, the assay values obtained agree very well with those obtained in the more customary assays employing *L. arabinosus* and recoveries of added nicotinic acid are good (95-97%).

The method has not been widely tested.

F. THIAMINE (YEAST GROWTH METHODS)

Despite the fact that thiamine was one of the first components of the bios complex to be identified (261), few yeast growth methods for determining this vitamin have been proposed. This was partially due to early development of a satisfactory method for thiamine based upon fermentation by yeast (see below), and partly because it was recognized that for most yeasts, one or both of the split products of thiamine could substitute for the intact vitamin. These products are not active for animals, and assays based upon yeast growth often yield higher values than are indicated by methods specific for the intact thiamine molecule. Both of the yeast growth methods proposed so far employ organisms for which thiamine split products are active. One yeast, *Rhodotorula aurantiaca*, has been found which requires *molecular* thiamine for growth (273), but assay methods based on use of this organism have not been developed.

(1) Assay Media and Their Preparation

The composition of assay media is given in Table XVIII. A stock solution of the basal medium containing all of the listed ingredients is prepared in each case. The hydrolyzed casein required for Medium 45 is prepared as described on p. 364. Thiamine-free yeast and liver extracts are not incorporated into the stock solution of Medium 45, but are added just preliminary to assay (see below). They are prepared in the following manner.

a. Preparation of Thiamine-Free Liver and Yeast Extracts for Medium 45. Five g. of Wilson's 1:20 liver extract are added to 195 cc. of water, autoclaved for 15 min. and cooled to room temperature. The pH is adjusted to approximately 3.0. Ten g. of fuller's earth are added, the mixture is shaken for 30 min., filtered, and the pH adjusted to approximately 1.0. After autoclaving again for 15 min. and cooling, a second 10 g. portion of fuller's earth is added and the mixture shaken for at least 2 days. At the end of this time it is filtered, the pH adjusted to 4.5-4.8, and again filtered. The filtrate is divided into 5 cc. (1 cc. equivalent to 2.5 mg.) portions and placed in test tubes which are plugged, autoclaved 15 min. at 15 lb. pressure and stored in the refrigerator until used.

The same procedure is followed for the yeast extract, with Bacto-Yeast Extract (Difco Laboratories, Detroit) as starting material.

(2) The Assay Methods

a. Method of Williams, McMahan and Eakin (297).

Assay organism—*Saccharomyces cerevisiae* O.P., ATCC No. 7753. Carried by weekly transfer on molasses-agar slants, with incubation at 30° C. for 24-36 hr., followed by storage in the refrigerator.

Basal medium—Medium 45, Table XVIII.

Sterilization—By steaming 10 min. at atmospheric pressure.

Inoculum—Yeast from a slant culture is suspended uniformly in a small volume of sterile basal medium, and its concentration determined turbidimetrically by use of an appropriate calibration curve. By calculation, enough of this suspension is added to the previously sterilized basal medium to bring the final concentration to 0.08 mg. of moist yeast /100 cc.

Procedure—Assays are carried out in ordinary chemical or bacteriological test tubes supported in a wire rack. All standard solutions and samples for assay are diluted with pH 4.7 acetate buffer (glacial acetic acid 3.75 g., anhydrous sodium acetate 5.0 g., water to 1 l.). These are added to assay tubes in volumes of 0.01 to 0.1 cc. 0, 0, 0.2, 0.4, 0.6, 0.8, 1.2 and 2.0 mγ of thiamine hydrochloride are appropriate amounts to establish the standard curve. 0.4 cc. of a buffered mixture of thiamine-free yeast and liver extracts (5 cc. of liver extract, 5 cc. of yeast extract, 50 cc. of acetate buffer) is now added to each tube, and all tubes are sterilized by steaming for 10 min. After cooling, 2 cc. of the inoculated basal medium are added to each tube.

Incubation—The unplugged tubes are incubated in the water bath at 30° C. for 15 hr. Tubes are chilled in the refrigerator, then each tube is diluted by addition of 5 cc. of saturated aqueous chlorothymol solution.

TABLE XVIII

Composition of Assay Media for Thiamine

Constituent	Medium No. 45. Williams, McMahan and Eakin, 1941 (297)	Medium No. 46. Woolley, 1941 (298, 299)
	Amount/100 cc. of basal medium	
Glucose	—	2.0 g.
Sucrose	2.0 g.	—
$(NH_4)_2SO_4$	300 mg.	300 mg.
KH_2PO_4	200 mg.	200 mg.
$MgSO_4.7H_2O$	25 mg.	25 mg.
$CaCl_2.2H_2O$	25 mg.	25 mg.
$ZnSO_4$	100 γ	100 γ
$MnCl_2$	100 γ	100 γ
$FeCl_3$	50 γ	50 γ
$CuSO_4.5H_2O$	10 γ	10 γ
KI	10 γ	100 γ
H_3BO_3, $TlCl_3$	100 γ	—
L-Asparagine	300 mg.	10 mg.
Inositol	500 γ	500 γ
Calcium pantothenate	30 γ	50 γ
Pyridoxine	2 γ	2 γ
Biotin	.02 γ	1 γ
Folic acid	.05 γ	—
Hydrolyzed casein, vitamin-free	20 mg.	—
Liver extract, thiamine-free	[a]	—
Yeast extract, thiamine-free	[a]	—

[a] Not included in stock solution of basal medium; see text.

Measurement of response—Turbidimetric.

Comment—Under the test conditions, the "thiazole" and "pyrimidine" portions of the thiamine molecule both have activity: 4-methyl-5-β-hydroxyethylthiazole is 60% as active, and 2-methyl-5-ethoxymethyl-6-aminopyrimidine is 30% as active, as thiamine. Treatment of thiamine with Na_2SO_3 under conditions which destroy all of the intact vitamin results in only 30% destruction of activity for yeast. Cocarboxylase, on the other hand is essentially inactive, so that samples must be suitably hydrolyzed before assay. Assay values obtained with the method are satisfactorily constant at increasing assay levels, and recoveries of thiamine added to tissue extracts varied between 88 and 107% (297).

From the activity of fragments of the thiamine molecule, which are known to occur naturally (*cf.* (300)), and are formed to some extent by procedures, such as heating, which inactivate thiamine, it could be predicted that this method would give high results for some foodstuffs. Cheldelin and Williams (301) reported good agreement in most instances between values obtained by this yeast growth procedure and those obtained by the thiochrome method; these comparisons were not made on the same samples, however. Heat-processed foods gave values by the yeast method which were much too high. Eppright and Williams (303) later compared the yeast growth method, the thiochrome method, and the yeast fermentation method by applying each procedure to the same samples. The yeast growth method gave higher values (from + 24 to + 720%) than the other two methods on almost all samples when enzymatic digests of the samples were assayed directly; especially high values were obtained with processed foods. The specificity of the method was increased by carrying out a preliminary adsorption of the thiamine on Decalso, followed by elution, as practiced commonly in certain thiochrome procedures (*e.g.*, 302). Assays on the eluate agreed much better with those performed by the thiochrome or fermentation procedures, but were still slightly higher in most cases.

b. Method of Woolley (298, 299).

Assay organism—*Endomyces vernalis*, strain or method of carrying stock culture not specified.

Basal medium—Medium 46, Table XVIII.

Sterilization procedures—Not specified; steaming for 10 min. at atmospheric pressure is satisfactory.

Inoculum—A transfer is made from a slant of the stock culture to a liquid medium consisting of the basal medium supplemented with the aqueous extract of 10 g. of malt sprouts/100 cc. Incubated 24 hr. at 30° C., cells collected by centrifugation, washed 3 times with sterile phosphate buffer, then suspended in a volume of buffer 20 times that of the original culture. One drop of this suspension is used to inoculate each assay tube.

Procedure—Samples and standard are added to 50 cc. Erlenmeyer flasks in amounts ranging up to 1.0 cc. The assay range is from 0.01 to 0.1 γ of thiamine per flask. All volumes are adjusted to 1 cc., and 10 cc. of the basal medium are added. After sterilizing and cooling, one drop of inoculum is added to each flask.

Incubation—At 37° C. for 24 hr.

Measurement of response—Turbidimetric.

Comment—The assay organism differs in specificity from that used by Williams, Eakin and McMahan (Method "a" above). The thiazole portion of thiamine is inactive; the pyrimidine portion has the same activity as thiamine on the molar basis. Cocarboxylase is as active as thiamine (molecular basis). The test organism grows as well in the basal medium plus thiamine as it does when plant extracts are added.

The method has not been applied to the routine estimation of thiamine in natural materials. Presumably, it suffers to a lesser degree from the same defect shown by Method "a" above, in that it will give too high results on materials which contain the free pyrimidine component of thiamine. If desired, this fault could probably be eliminated by a correction procedure similar to that employed in the yeast fermentation method described below.

G. THIAMINE (YEAST FERMENTATION METHOD)

All of the methods previously considered in this chapter have involved measurement of the growth response to a vitamin, either directly or indirectly, through measurement of a metabolic product which accumulates during growth. A fundamentally different type of microbiological assay was devised for thiamine and successfully employed as early as 1937 by Schultz, Atkin and Frey (304). This method was based upon the observation that, in the presence of ammonium phosphate buffer, thiamine greatly increased the rate of fermentation of sugar by yeast, under conditions where no growth occurred. The rate of fermentation was conveniently measured by determining the volume of CO_2 evolved. A subsequent report (305) gave details of the method and showed that the pyrimidine moiety of thiamine was approximately as effective as thiamine itself in stimulating gas production. Later, it was shown that inclusion of nicotinic acid in the fermentation medium increased the specificity (306); and that the effect of thiamine could be differentiated from that of its pyrimidine component by determining the total effect of both stimulants, then destroying the thiamine specifically by a selective oxidation to thiochrome (307, 308), or by sulfite cleavage (309), and redetermining the effect of the pyrimidine component alone. Thiochrome, and thiazole and the pyrimidine sulfonic acid arising from thiamine by sulfite cleavage are inactive in the fermentation test. The complete improved procedure, together with a simplified instrument for use with it, were described in 1942 (310). This procedure will be given below.

(1) Method of Schultz, Atkin and Frey (310).

a. Principle of the Method. Two preparations are made of each sample to be analyzed—one treated with sulfite to destroy thiamine, and one untreated. Fermentation activities of each are then determined in the fermentometer. The difference, expressed in terms of pure thiamine chloride, is taken as a measure of the thiamine content of the sample.

b. Preparation of samples (*cf.* also discussion of this on p. 372).

The finely ground or blended material is dissolved or suspended in a convenient volume of water, and H_2SO_4 is added until the solution is acid to Congo red paper. It is then heated at 100° C. for 20 min. When cool, the sample is diluted to volume and a portion taken for sulfite treatment (see below). The remainder is neutralized to litmus with NaOH and is then used for determination of total fermentation activity, *i.e.*, before sulfite treatment.

c. Treatment with Sulfite. An aliquot of the above sample, taken before neutralization, and not exceeding 20 cc. in volume is placed in a 50 cc. Erlenmeyer flask. Seven-tenths cc. of N H_2SO_4 and 5 cc. of a freshly prepared solution of Na_2SO_3 containing 0.2 g. of $Na_2SO_3 . 7H_2O$ /5 cc. are added. The reaction is adjusted to pH 5.2-5.6 with dilute H_2SO_4 or NaOH (bromcresol purple is a convenient outside indicator). The flask is capped with a 30 cc. beaker, heated at 100° C. for 30 min. and then cooled. Excess sulfite is destroyed by the addition of the exact quantity of a 3% solution of H_2O_2. The end-point of this reaction is determined by means of an outside indicator made by mixing one drop of 5% KI, one drop of 1% soluble starch indicator, and one drop of 50% H_2SO_4. The mixture develops a pink tinge which gets darker with time. If excess sulfite is present, the pink color is discharged; at the end-point, no color change appears, and if excess H_2O_2 is present a deep violet color appears.[49]

When the excess sulfite has been destroyed, the solution is neutralized to litmus, made to volume, and an aliquot portion used for the determination of residual fermentation activity. Control experiments with pure thiamine show that over 99% destruction results from sulfite treatment carried out under these conditions of pH and temperature. When applied to natural materials, 99% of the products stimulatory to fermentation are seldom destroyed, apparently due to the presence, in small amounts, of the pyrimidine component of the thiamine molecule. In the majority of cases, this residual activity or "sulfite blank" amounts to 10%, or less, of the total fermentation-stimulating activity. Heat-treated or processed foods may, of course, show considerably higher proportions, due to hydrolytic destruction of thiamine during processing.

d. Reagents.

Solution A—One liter contains 180 g. of $NH_4H_2PO_4$, 72 g. $(NH_4)_2$-

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[49] Using a micro fermentation procedure (311), Josephson and Harris found that traces of undestroyed sulfite inhibited subsequent fermentation. They recommend use of a slight excess of H_2O_2—1 or 2 drops—so that a distinct purple color of the indicator will develop. Eppright and Williams (303) found this procedure also aided obtention of satisfactory replicate values for sulfite-treated samples in the macro procedure.

HPO_4, and 0.2 g. of nicotinic acid. The solution should be sterilized by heating at 100° C. (Arnold sterilizer) for 30 min. It may be transferred to a sterile automatic burette and kept at room temperature, or handled in the same manner as solution B.

Solution B—One liter contains 200 g. of anhydrous dextrose, 7.0 g. of $MgSO_4.7H_2O$, 2.2 g. of KH_2PO_4, 1.7 g. of KCl, 0.5 g. of $CaCl_2.2H_2O$, 0.01 g. of $FeCl_3.6H_2O$, and .01 g. of $MnSO_4.4H_2O$. It is distributed in 500 cc. Erlenmeyer flasks, cotton-plugged, and sterilized at 100° C. on 3 successive days. Thereafter, flasks may be kept at room temperature if they are capped with foil or paper to prevent evaporation. After a flask has been opened for use, it is kept in a refrigerator at 4° C. until empty.

Thiamine standard solution—Crystalline thiamine chloride is dried for 24 hr. over P_2O_5 in a desiccator. A weighed amount is dissolved in distilled water to make a solution containing 0.1 mg./cc. This solution is distributed in 200 cc. Erlenmeyer flasks (cotton-plugged), sterilized at 100° C. for 20 min. on 2 successive days, then treated like solution B.

Yeast suspension—Ten g. of Fleischmann's baker's yeast or its equivalent is weighed into a deep scoop or a small beaker; distilled water is added, slowly at first, until a smooth, heavy cream has been produced. This is then diluted with distilled water to 200 cc. This suspension is made just prior to each run. The small foil-wrapped cakes of yeast or the high vitamin B_1 baker's yeast should not be used.

e. Apparatus. The fermentometer required consists of a water thermostat, a shaking device and a series of 12 gasometers. Reference should be made to the original article for a description of it. The instrument is available from commercial supply houses.

f. Procedure. (a) Preliminary—Test runs should be made with the apparatus to be certain it is working properly. In satisfactory operation, duplicate determinations should agree within 2%. Uniform temperature, freedom from leaks, accurately calibrated gasometers are necessary. The shaking rate should be such that no yeast settles from suspension, nor should the reaction mixture be violently thrown against the stopper. In a 3 hr. test run the difference in CO_2 evolution produced by 1 and 2 γ of thiamine should not be less than 20 cc. and preferably 30 cc. The samples should be assayed at a level which contains between 1 and 2 γ of thiamine; if this requires unduly large amounts of the sulfite-treated sample, 1 γ of thiamine may be added to bring this into the assay range. Each sample requires two reaction bottles, one for the untreated extract and one for the sulfite-treated extract (the "sulfite blank"). The standard is also run in two bottles, one containing 1 γ of thiamine, the other containing 2 γ. (b) Assay—In each reaction bottle, 2.5 cc. of solution A and 7.5 cc. of solution B are placed, followed by addition of the vitamin-containing solutions (1 and 2 γ of thiamine for the standard bottles, amounts of sample solutions containing between 1 and 2 γ of thiamine for each sample (see (a) above)). The contents of each bottle are diluted to 40 cc. The time is noted, and 10 cc. of the yeast suspension containing 0.5 g. of moist compressed yeast, are added to each bottle as rapidly as possible. A free-flowing pipette is desirable for this purpose.

As quickly as possible, the reaction bottles are placed in the fermentometer and connected to the gasometers. Shaking is started. After 2-3 min., the initial reading of the gas volumes is made. After 3 hr. additional shaking, the final readings of gas volumes are made. The difference represents the gas evolved.

g. Calculation of Results. In the limited range used, a linear relationship exists between the thiamine present and the gas evolved, so that calculations are very simply made. Examples are given in Table XIX.

h. Comment. This procedure for thiamine determination has been widely used and discussed (cf. 311a) and appears to be among the most satisfactory of the methods available. Results have been shown to agree well, in most cases, with results of other methods (312, 303, 313, 314). Despite the general satisfactoriness, certain samples, as in most methods, give trouble. Thus, alkali-treated materials and urine samples appear to give considerably higher values by this procedure than by the thiochrome or yeast growth methods, despite correction for the sulfite blank (303), and stimulatory materials of similarly unknown nature may occur in other natural samples. Deutsch (315) using a micro modification of the method (316) found that both the thiazole and the pyrimidine-sulfonic acid which result from sulfite cleavage of thiamine exerted a stimulatory effect on fermentation, the magnitude of which varied with the level tested. Together, in equivalent concentration, their activity approximated 22.3 to 14.4% of the activity of the thiamine from which they arose by sulfite cleavage. Scrimshaw and Stewart (317) noted similar results in the macro fermentation procedure, but not to a troublesome degree. These authors (317) also found that, with some products (specifically meat and egg products), thiamine added to the basal medium alone showed less activity than it did in the presence of the sample or its sulfite blank. This tended to produce high assay values. Also, the range of linear response to added thiamine varied with the sample assayed and the yeast used. The following modifications of the procedure of Schultz *et al.*, described above, were suggested to eliminate these 3 potential errors;

(1) For each type of substance assayed, a preliminary run is made in which graded amounts of crystalline thiamine are added to the sulfite blank. The gas evolution is noted, and the range in which gas evolution

TABLE XIX

Sample Data and Calculations for Determination of Thiamine by Yeast Fermentation

Bottle No.	Addition to Control Reaction Mixture	Gas evolved in 3 hr.	Activity in terms of thiamine	Apparent thiamine content	Free thiamine content
		cc.	γ	γ/g.	γ/g.
1	Thiamine chloride, 1 γ	170	1	—	—
2	" " , 2 γ	200	2	—	—
3	Sample A, untreated, 10 mg.	190	1.67	167	155
4	Sample A, sulfite-treated, 100 mg.	175	1.17	11.7	0.0
5	Sample B, untreated, 20 mg.	182	1.40	70	67
6	Sample B, sulfite-treated, 100 mg. + 1 γ thiamine	179	1.30	3.0	0.0

is linearly related to thiamine concentration is used for subsequent assays of that type of material. This insures that the assay will be run at a level where the response to added thiamine is linear.

(2) A standard 3-bottle assay (in duplicate, if desired), is run, in which one bottle contains the sample, one the sulfite-treated sample, and the third the sulfite-treated sample plus a suitable amount of crystalline thiamine (as determined in (1)).

(3) The amount of gas produced by the known amount of thiamine in the presence of the sulfite-treated sample is used for the calculation of the thiamine content of the sample, thus eliminating the error due to enhancement of response to thiamine by constitutents of the sample.[50]

(4) The effectiveness of the sulfite cleavage of a known amount of thiamine added to the sample is determined for each type of substance assayed, and a correction made for the activity of the cleavage products, if necessary. With the samples tested, the sulfite procedure was found very satisfactory; activity greater than 8% of the original was not encountered in any of the sulfite-cleaved samples studied. With these modifications, the fermentation procedure, in their hands, gave excellent results.

An ultramicro modification of the yeast-fermentation procedure which employs special flasks with the Warburg apparatus, and which is applicable in the assay range from 10 to 20 mγ of thiamine has been described by Atkin, Schultz and Frey (316), and later modified by Josephson and Harris (314). The original papers should be consulted for details of these procedures.

H. GENERAL METHODS FOR ASSAY OF VITAMINS WITH YEAST

As stated earlier, yeasts are known which require 0 to 6 different vitamins. It would appear possible, at present, to devise a single assay medium, which, by omission of the proper vitamin, would permit estimation of all of the vitamins required by a given organism. The procedures of Atkin and his coworkers for determination of biotin (282), inositol (282), pantothenic acid (30), and vitamin B_6 (221), approximate such a general procedure, except for minor variations in medium and technique which may or may not be significant. Burkholder (267) has shown that the yeast *Kloeckera brevis* requires nicotinic acid, vitamin B_6, thiamine, pantothenic acid, biotin, and inositol, and has presented curves relating growth to concentration of each of these 6 vitamins, obtained with a single basal medium. The procedure has not been applied to natural materials, but could, perhaps, be adapted into an excellent general method for these 6 vitamins. Such general procedures would have many advantages over the several slightly different methods which are now in use.

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[50] It is assumed that the sulfite-treated sample has the same enhancing effect, if any, shown by the untreated sample. With the samples investigated (317), this was the case.

IV. Methods Utilizing *Neurospora* Mutants as Assay Organisms

1. THE NUTRITIVE REQUIREMENTS OF NEUROSPORA

Butler, Robbins and Dodge (318) and Beadle and Tatum (319) showed, in 1941, that naturally-occurring, wild-type strains of *Neurospora* required biotin as the sole growth factor of vitamin-like nature, and that, if this were supplied, these organisms grew well on a medium containing a carbon and energy (*e.g.*, sucrose) source, an inorganic nitrogen source, and several inorganic salts. The present interest in these organisms and their application to microbioassay stem from the now classical observations of Beadle and Tatum (319) that, by X-ray irradiation, mutant strains could be derived which were unable to synthesize either thiamine or vitamin B_6, and therefore required addition of one of these vitamins to the basal medium. Since these initial observations, many additional mutants have been obtained, which require one or more of a variety of vitamins, amino acids, and other substances (320). Some, but by no means all, of these mutant cultures have been applied to the determination of the substances which they require. In a recent publication, Beadle and Tatum (320) list mutants which require thiamine, riboflavin, vitamin B_6, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, inositol, and choline. Since the parent culture, and all of the mutants require biotin, all of the known B vitamins, folic acid excepted, could be determined in this fashion if desired. Methods so far suggested which use these organisms will be considered below.

(2) Assay Methods for Individual Vitamins

In the methods to be described below, the practice followed earlier in describing assays with lactic acid bacteria and with yeasts will be followed, *i.e.*, the first methods will be described in considerable detail, and subsequent procedures summarized in less detail. Extraction procedures for the various vitamins have been summarized earlier (see section on "Methods Utilizing Lactic Acid Bacteria as Assay Organisms"), no mention of these will be made with these methods except in the case of choline, which has not been considered previously.

A. *p*-AMINOBENZOIC ACID

(1) Method of Thompson, Isbell and Mitchell (248)

a. Assay Medium and Its Preparation. The composition of the assay medium is given in Table XX. Except for the crude supplements, it is the same as that suggested earlier by Tatum and Beadle (243). The supplements are prepared in the following manner.

(1) *Acid-hydrolyzed, charcoal-treated casein.* Labco vitamin-free casein, (50 g.) is mixed with 500 cc. of 6 *N* HCl, and the mixture refluxed for 10 hr. HCl is removed by repeated vacuum distillation, the pH adjusted to 3.0, and the volume to 500 cc. Five g. of decolorizing charcoal (Darco G-60) are added; the mixture is shaken 15 min., then filtered to remove charcoal. The pH is adjusted to 7.0 and the solution preserved under toluene.

(2) *Enzymatic digest of beef liver and muscle.* Ten g. of fresh, finely ground beef liver or muscle are suspended in 50 cc. of acetic acid-sodium acetate buffer, pH 4.5 (sodium acetate 5.0 g., glacial acetic acid, approximately 3.8 g., water to 1 l.). To this mixture is added 0.1 g. each of clarase (Takamine Laboratories) and caroid (a papain preparation of the American Ferment Company). The mixture is incubated under benzene (temperature not specified, presumably 37° C.) for 24 hr. The benzene is then removed by steaming and the solids by filtration.

(3) *Charcoal-treated yeast extract.* Ten g. of yeast extract (Bacto, from Difco Laboratories) are dissolved in 100 cc. of water. The pH is adjusted to 3.0, 2 g. of activated charcoal (Darco G-60) are added, and the mixture is shaken for 15 min. The charcoal is filtered out and the pH adjusted to 7.0.

(4) *Agar.* Commercial agar may contain appreciable amounts of *p*-aminobenzoic acid, and is, therefore, washed thoroughly. The agar is uniformly suspended in a large volume of distilled water, allowed to settle, and the water decanted. This procedure is repeated 15-20 times during a period of 24-48 hr.

The washed agar, casein hydrolyzate, and enzymatic digests of beef liver are added to the medium just before use.

b. The Assay Method.

Assay organism and inoculum—*Neurospora crassa "p-aminobenzoic-less,"* No. 1633 ATCC No. 9278. Carried on agar slants of the basal medium supplemented with 0.05 γ of *p*-aminobenzoic acid per tube (*ca.* 10 cc.), incubated at 30° C. or room temperature. To prepare inoculum, a wire loop is moistened and used to collect a loopful of spores from a slant of the stock culture. This is thoroughly dispersed in 1-2 cc. of sterile liquid basal medium supplemented with 5 mγ of *p*-aminobenzoic acid. This agar medium, with suspended spores, is poured into a sterile Petri dish and incubated 16 hr. at 30° C. It may be kept in the refrigerator for a short period, if necessary, before use. In the incubated culture, the spores have germinated and the incipient mycelia, which are just visible to the naked eye, should be distributed uniformly throughout the agar. Blocks of agar cut from this plate are used for inoculating plates containing the sample to be assayed. These blocks are easily removed with the aid of a sterile 8 mm. cork borer fitted with a plunger for extruding the agar block. Care must be taken to secure circular blocks of uniform thickness, as any irregularity in the shape of the inoculum block is reflected in the subsequent growth of the mold.

Basal medium—Medium 47, Table XX.

TABLE XX

Composition of Assay Medium for Determination of p-Aminobenzoic Acid with Neurospora crassa

Medium No.

47. Thompson, Isbell and Mitchell, 1943 (248)

Constituent	Amount/100 cc. of basal medium [a]	Constituent	Amount/100 cc. of basal medium [a]
Ammonium tartrate	0.5 g.	Agar (washed)	2.0 g.
KH_2PO_4	0.1 g.	Casein, acid-hydrolyzed, vitamin-free	0.1 g.
NH_4NO_3	0.1 g.		
$MgSO_4 \cdot 7H_2O$	50 mg.	Enzymatic digest of beef liver	50 mg.
NaCl	10 mg.		
$CaCl_2$	10 mg.	Enzymatic digest of beef muscle	10 mg.
$FeCl_3$	0.5 mg.		
Biotin	0.4 γ	Yeast extract, charcoal-treated	10 mg.
Sucrose	1.5 g.		

[a] The basal medium is 1.1 times its final concentration.

Procedure—0-1 cc. of the standard *p*-aminobenzoic acid solution and solutions for analysis are pipetted into test-tubes and diluted, where necessary, to 1 cc. Standard tubes should contain from 4 to 40 mγ of *p*-aminobenzoic acid to permit construction of an adequate standard curve; samples should be assayed within this concentration range. Fifteen cc. of the hot basal medium are now added to each tube, and these are then autoclaved at 15 lb. pressure for 15 min. Immediately after removal from the autoclave, the contents of the tubes are transferred to sterile Petri plates. The Petri plates used for this purpose should have uniform bottoms, thus giving an agar layer of uniform depth. Molded soft glass plates were found most satisfactory in this respect. When the agar has solidified, an inoculum block is placed upon the surface in the center of each plate. These are incubated right side up (to prevent the mold from growing downward) at 30° C. for approximately 20 hr.

Measurement of response—The diameter of the mold growth surrounding the inoculum block, which is quite uniformly circular, is measured with calipers, and is dependent upon the amount of *p*-aminobenzoic acid in the culture plate. Diameter of growth is plotted against concentration of *p*-aminobenzoic acid to produce the standard curve, from which the *p*-aminobenzoic acid content of the sample aliquot is read in the customary manner.

Comment—The assay medium is not completely free of *p*-aminobenzoic acid, due to the inclusion of beef liver and muscle digests, but is very low in this substance. The diameter of mycelial growth may vary from about 35 mm. (with no *added* *p*-aminobenzoic acid) to 65 or 70 mm. with added *p*-aminobenzoic acid. No tested compound related to *p*-aminobenzoic in structure has activity over a fraction of 1% of that of *p*-aminobenzoic acid (243). Recoveries of the added vitamin are quantitative to within ± 10%. Reproducibility of results on natural materials is stated to be about ± 15%. Samples for assay must not contain large amounts of salt, which are toxic to the assay organism; the samples are best prepared for assay by hydrolysis with H_2SO_4, as discussed on p. 414.

The procedure depends on measuring the *rate* of growth of the test organism, as determined by the concentration of *p*-aminobenzoic acid in the medium. It differs in this respect from most other quantitative methods developed for these organisms, in which the *amount* of growth is measured after it has proceeded approximately to completion.

The pH of the medium used above is not stated by the authors. For maximum sensitivity, this should be adjusted to 4.0, since it has been shown (321) that the activity of *p*-aminobenzoic acid for this organism is dependent upon the pH, and increases markedly as the pH is lowered from 7.0 to 4.0. For this reason, samples should be adjusted to the pH of the medium before assay if the amounts used are sufficient to change the pH of the medium significantly.

This method is illustrative of methods which depend upon measurement of the rate of growth. Rate measurements can be made equally well by growing the culture in special horizontal tubes half-filled with the supplemented basal medium, and inoculated at one end. The position of the growing frontier is then marked on the tube at various times (322). In general, methods which depend upon rate measurements of this kind are less precise than those in which the total amount of growth permitted by a given amount of an essential substance is measured (323). Most of the quantitative methods developed for *Neurospora* have been of the latter type. The customary type of *Neurospora* assay, in which mycelial weights are determined, has also been used for determination of p-aminobenzoic acid by Tatum, *et al* (324a), and is discussed below.

(2) Method of Tatum, Ritchey, Cowdry and Wicks (324a)

See under section F, p. 468, dealing with general methods.

B. VITAMIN B_6

(1) Method of Stokes, Larsen, Woodward and Foster (223, 323a, 324)

a. Assay Medium and Its Preparation. The composition of the medium is given in Table XXI. Except for inclusion of zinc sulfate, the medium is very similar to that of Tatum and Beadle (243). The zinc represses sporulation of the mold, and thus facilitates subsequent harvesting of the mycelium. It is convenient to prepare stock solutions of the various mineral ingredients of the medium, and combine these in appropriate amounts to prepare the final medium. A slight cloudiness which may appear does not interfere with the determination.

b. The Assay Method.

Assay organism and inoculum—*Neurospora sitophila "pyridoxinless,"* No. 299 ATCC No. 9276. Cultures are grown on Saboraud agar slants (maltose 38 g., Bacto-peptone 8 g., Bacto-malt extract 2 g. agar 20 g., water 1 l.) at 30° C until sporulation, which usually occurs in 4 or 5 days and is evidenced by the pink or orange coloration appearing on the upper portion of the slant. After sporulation, cultures are stored in the refrigerator until used. Fresh cultures are prepared every 3 weeks. Inoculum is prepared by removing a small portion of growth, about 3 mm. in diameter, and consisting primarily of spores, from a slant culture with a sterile platinum loop, and suspending this uniformly in 10 cc. of sterile water. Each assay flask is inoculated with 1 drop of this inoculum.

Procedure—Five cc. quantities of the basal medium (No. 48, Table XXI) are placed in 50 cc. Erlenmeyer flasks. Up to 5.0 cc. of standard pyridoxine solution or sample extract (see below) are next added, in duplicate, and the total volume adjusted with distilled water to 10 cc. Concentrations of pyridoxine of from 0.1 to 1.0 γ are suitable for establishing the standard curve. Flasks are plugged with cotton, autoclaved at 15 lb. pressure for 15 min., allowed to cool, then inoculated with 1 drop of the inoculum suspension. The inoculated flasks are incubated at 30° C. for 5 days, during which time a mat of fungal mycelium develops on the surface in amounts depending, within limits, on the amount of vitamin B_6 present.

Determination of response—After the incubation period, the cultures are steamed at 100° C. for 5 min., and the mycelium harvested. It is easily removed from the flask in one piece with a stiff wire needle. Bits of mycelium that have grown up the sides of the flask are wiped up with the main portion of the mycelium. The fungus growth is pressed dry between paper towels or filter paper, rolled into a small pellet, dried at 100° C. for 2 hr., then weighed to the nearest mg. on an analytical balance. Glazed porcelain spot plates are convenient for handling the mycelium during drying and weighing. A standard curve relating concentration of pyridoxine to weight of mycelium is constructed, and the amounts of vitamin B_6 present in the sample aliquots determined from it in the customary fashion.

TABLE XXI

Composition of Medium for Assay of Vitamin B_6 with Neurospora sitophila

Medium No.

48. Stokes, Larsen, Woodward and Foster, 1943 (223)

Constitutent	Concentration /100 cc. of basal medium [a]	Constitutent	Concentration /100 cc. of basal medium [a]
Sucrose	3.0 g.	NaCl	20 mg.
NH_4 tartrate	1.0 g.	$CaCl_2$	20 mg.
KH_2PO_4	0.5 g.	$FeCl_3$	1 mg.
$MgSO_4.7H_2O$	0.1 g.	$ZnSO_4.7H_2O$	0.2 mg.
		Biotin	0.8 γ

[a] The medium as given is twice its final concentration.

Preparation of the sample—Procedures for extraction of vitamin B_6 have been considered earlier, p. 408. In this method, however, presence of small amounts of thiamine stimulates growth of the test organism in the presence of suboptimal amounts of pyridoxine. Thiamine is, therefore, destroyed in each sample before assay[51] by a modification of a sulfite cleavage procedure (310). To 10 cc. of the extract (derived from 0.2 to 1.0 g. of sample) is added 0.7 cc. of 10 *N* NaOH, followed by 5 cc. of a freshly prepared 1% solution of anhydrous Na_2SO_3. The pH of the mixture is adjusted to 8.0 with cresol red as an outside indicator. The mixture is steamed at 100° C. for 30 min., cooled, and treated with the exact quantity of a fresh 2% H_2O_2 solution necessary to destroy the excess sulfite. (See p. 453 for a description of this technique.) The sample is then ready for assay.

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[51] It is difficult to see why addition of an excess of thiamine to the basal medium should not correct this difficulty just as satisfactorily and with less effort than the procedure recommended by the authors. This procedure has recently been recommended by Tatum, *et al* (324a), whose procedure is summarized below.

Comment—The original paper and subsequent work (324) shows that if the pH of the medium rises above 5.8, the assay organism grows in the absence of vitamin B_6 and synthesizes it. The assay medium is, therefore, buffered at pH 5.0, and samples should not be used in amounts which shift the pH above 5.8. This is easily prevented by adjusting the pH of samples to 5.0 before assay.

The organism responds equally to pyridoxal, pyridoxamine, and pyridoxine, and is, therefore, suitable for the determination of the total vitamin B_6 content of natural materials (225). The assay appears to be reliable as judged by the following counts (223): (a) assay values obtained from different dosage levels of test samples agree closely, indicating absence of stimulatory or inhibitory substances; (b) different assays yield excellent duplication of values for the same materials; (c) recoveries of pyridoxine added to samples are quantitative within ± 15%; and (d) values obtained for a variety of products check closely values obtained by animal assay on the same or similar products.

A somewhat similar but apparently less suitable assay procedure using the same test organism has been studied by Bonner and Dorland and applied to determination of the distribution of vitamin B_6 in the tomato plant (325). An alternative assay method with this organism described by Tatum, *et al.* (324a) is somewhat simpler to carry out, and is more sensitive. By addition of excess thiamine to the medium, the sensitivity of the organism to vitamin B_6 is increased almost 15-fold, and the necessity for treating samples with sulfite to destroy thiamine is eliminated. This method is described below. Its accuracy has not been checked so thoroughly as that of Stokes, *et al.*, above.

(2) Method of Tatum, Ritchey, Cowdry and Wicks (324a)

See under section F, p. 468, dealing with general methods.

C. CHOLINE

(1) Method of Horowitz and Beadle (326)

a. Assay Medium and Its Composition. The composition of the basal medium is given in Table XXII. It is made up without sucrose or biotin, autoclaved, and stored until use. For convenience in storing, it may be

TABLE XXII

Composition of Medium for Assay of Choline with Neurospora crassa

Medium No.

48a Horowitz and Beadle, 1943 (326)

Constituent	Concentration /100 cc. of basal medium [a]	Constituent	Concentration /100 cc. of basal medium [a]
Sucrose	2.0 g.	Biotin	0.5 γ
NH_4 tartrate	0.5 g.	Trace elements [b]	
NH_4NO_3	0.1 g.	B	1 γ
KH_2PO_4	0.1 g.	Mo	2 γ
$MgSO_4.7H_2O$	50 mg.	Fe	20 γ
NaCl	10 mg.	Cu	10 γ
$CaCl_2$	10 mg.	Mn	2 γ
		Zn	200 γ

[a] The medium is from 1 to 1.2 times its final concentration (*cf.* text).

[b] Added as salts

prepared 3 times as concentrated as indicated, then diluted and supplemented with sucrose and biotin just before use.

b. The assay method.

Assay organism and inoculum—*Neurospora crassa "cholineless,"* No. 34486; ATCC No. 9277. Carried on agar slants composed of the basal medium plus 1.5% agar, 0.2% Difco yeast extract, 0.2% malt extract, and 1 γ/cc. of choline. For inoculum a spore suspension is made in a few cc. of sterile water, and 1 drop of this is used for each assay flask.

Procedure—Aliquots of standard solution containing 0-20 γ of choline are placed in 250 cc. Erlenmeyer flasks. Sample aliquots containing 0.5-20 γ of choline, and prepared as described below, are similarly distributed among flasks, with each concentration in duplicate. Not more than 5 cc. of standard or sample solution should be used. Basal medium (No. 48, Table XXII) is now added to bring the volume in each flask to 25 cc. The flasks are autoclaved at 15 lb. pressure for 5-10 min., cooled, inoculated, and incubated at 25° C. for 3 days.

Measurement of response—The dry weight of mycelium is determined as described under the method for vitamin B_6, above. Siegel (327) recommends filtering the contents of each assay flask through tared, fritted glass filters (30 cc.) of medium porosity, washing the mycelial mat with water, and drying to constant weight for most accurate results.

Preparation of sample—Combined choline, as in lecithin, is slowly utilized by the test organism, and must be liberated by hydrolysis.

Methionine is 0.002 as active as choline, and interferes with choline assay when present in excess of 0.1 mg./25 cc. of culture medium. In such cases, it must be removed from the sample. To accomplish these ends, the following procedure was developed.

One hundred mg. of the dry material to be analyzed are weighed into a 50 cc. Erlenmeyer flask, followed by 10 cc. of 3% H_2SO_4.[52]. The flask is plugged with cotton and autoclaved at 15-17 lb. for 2 hr. After cooling, the contents are transferred quantitatively to a 50 cc. Pyrex centrifuge tube, neutralized to Congo red with saturated $Ba(OH)_2$, centrifuged, and the supernatant filtered through a Whatman No. 50 paper. Three cc. of distilled water are added to the precipitate in the centrifuge tube and the contents brought to a boil, with stirring. After cooling and centrifuging, the washings are added to the previous supernatant. The clear filtrate is neutralized to litmus with *N* NaOH, and brought to a convenient volume, usually 30 cc., with distilled water.

To eliminate methionine [53] which may be present, 5 cc. of the neutralized solution (containing not less than 15 γ of choline) are run through a column measuring 110 × 0.6 mm., and containing approximately 1 g. of permutit (Permutit according to Folin, obtained from Coleman and Bell Co., Norwood, Ohio). The design of the columns is given by Dubnoff and Borsook (329). Such a column will adsorb quantitatively as much as 2.5 mg. of choline from 5 cc. of solution; methionine is not adsorbed. The column is then washed with 5 cc. of 0.3% NaCl; filtrate and washings are discarded. A test tube marked at 10 cc. is now placed under the column, and the choline is eluted with 10 cc. of 5% NaCl. The filtrate is brought to 10 cc. with distilled water, and appropriate aliquots used for the assay.

Comment—Dry weights of mycelium obtained range from 0 to about 60 mg. per flask. Dry weights from duplicate flasks agree, on the average, within 5%, while choline values determined on different amounts of the same solution agree generally within 10%. Recoveries of added choline range from 90 to 110% of theoretical. Assays show good day to day reproducibility, but standard curves obtained on different days, though similar, are not superimposable, and must be run with each assay.

Luecke and Pearson (330, 328) have used the method for the determination of free choline in biological materials. Comparative determinations on the same samples showed that microbiological values were in excellent agreement with those obtained by the chemical methods of Engel (331) and Marenzi and Cardini (332).

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[52] Luecke and Pearson (328) found 3 *N* HCl as satisfactory as 3 *N* H_2SO_4 obviating the necessity for removing $BaSO_4$. They also found 1 γ of biotin/l. of medium as satisfactory as 5 γ.

[53] Methionine is often times not present in interfering amounts; however, the authors recommend the permutite treatment to eliminate all non-basic substances which may inhibit or stimulate growth of the mold.

In their initial investigations, Horowitz and Beadle found only choline and methionine to be active. Lecithin was also active, but its choline was assimilated much less rapidly than was free choline. Betaine, creatine, sarcosine, ethanolamine, dimethylamine, trimethylamine, and tetramethyl ammonium hydroxide all were inactive. Jukes and Dornbush (333) found that β-dimethylaminoethanol was just as effective as choline as a growth factor for the organism; diethylaminoethanol was less than 0.001 as effective.

The procedure has been modified and simplified somewhat for use with milk products by Hodson (334). The essential features in which the modified procedure, which can also be used for determination of biotin, differs from the parent procedure, are given below.

c. The Modified Procedure of Hodson (334). This method was used and found satisfactory for milk products. The only changes have been to substitute 50 cc. flasks for 250 cc. flasks. Samples and standards are added as before in amounts up to 5 cc., all volumes are adjusted to 5 cc., and 5 cc. of *double strength* medium are added. Cultures are incubated for 5 days instead of 3. Only the steep portion of the standard curve is used. In preparing samples, the permutit treatment was omitted, since it was found unnecessary for milk products.

(2) Method of Tatum, Ritchie, Cowdry and Wicks (324a)

See under section F, p. 468, dealing with general methods.

D. BIOTIN

As stated before, all *Neurospora* cultures require biotin, and could, therefore, be used for its determination. Hodson and Norris (334) have used *Neurospora crassa "cholineless,"* with conditions and media described immediately above, for this determination, and obtain satisfactory results. Biotin is omitted from the medium, choline is added at a concentration of 2.0 mg./100 cc. of *double strength* medium.

(2) Method of Tatum, Ritchie, Cowdry and Wicks (324a)

See under section F, p. 468, dealing with general methods.

E. INOSITOL

(1) *Method of Beadle (335)*

a. Assay Medium and Its Composition. The assay medium used is identical with that used for assay of choline (Table XXII).

b. The Assay Method.

Assay organism and inoculum—*Neurospora crassa "inositolless,"* No. 37401; ATCC No. 9683. Stock cultures and inocula are prepared as described under choline assay, above. Inocula should be roughly standardized at a low level, and conidia be taken from stock cultures of a uniform age of 4, 5 or 6 days.

Procedure—Similar to choline assay, with the following differences. Cultures are grown in a total volume (sample plus medium) of 20 cc. in 125 cc. Erlenmeyer flasks. The assay range is from 5 to 30 γ of inositol per flask. Bound forms of inositol occur which are inactive. Of the procedures investigated, hydrolysis with 18% HCl for 6 hr., as recommended on p. 439, was found most effective.

Measurement of response—Determination of dry weight of mycelium (see choline and vitamin B_6 assays, above). 40-50 mg. of mycelium are obtained in flasks supplemented with optimal amounts of inositol.

Comment—The method gives, with most materials investigated (335), values which are satisfactorily constant at increasing assay levels. Yeast extract, corn meal, and some other materials give values which drift slightly, with high values at low assay levels. Recoveries of inositol are satisfactory. Indirect comparisons with the results of yeast assay (different samples), indicate that the two different types of procedures give the same results.

(2) *Method of Tatum, Ritchie, Cowdry and Wicks (324a)*

See under section F, p. 468, dealing with general methods.

F. GENERAL METHOD FOR p-AMINOBENZOIC ACID, BIOTIN, CHOLINE, INOSITOL AND VITAMIN B_6

(1) *Method of Tatum, Ritchie, Cowdry and Wicks (324a)*

a. Assay Medium and Its Composition. The assay medium is identical with that used for assay of choline (Medium 48a, Table XXII). For p-aminobenzoic acid, choline and inositol this medium (made up at double the concentration indicated) is used unchanged. For assay of biotin, the biotin of the medium is omitted, and the sucrose is purified by treating it (in 20% solution) with 0.1 of its weight of charcoal. For assay of vitamin B_6, an excess of thiamine (3γ per 10 cc.) is added to the medium; this eliminates the necessity for destroying thiamine. in the samples added, and increases the sensitivity of the method.

b. The Assay Method.

Assay organisms and inocula.—An appropriate mutant of *Neurospora* which requires the vitamin to be assayed, is used for each assay. These are listed below, together with the range of concentrations of the vitamins for establishing the standard curves.

Vitamin to be determined	Strain of *Neurospora* used	Effective vitamin range per 10 cc. of medium
Biotin	*Neurospora crassa* 1	0.00005 — 0.001 γ [a]
p-Aminobenzoic acid	*Neurospora crassa* 1633	0.01 — 0.5 γ [b]
Choline	*Neurospora crassa* 34486	0.5 — 5.0 γ [b]
Inositol	*Neurospora crassa* 37401	2.0 —10.0 γ [b]
Vitamin B_6 (pyridoxine + pyridoxal + pyridoxamine)	*Neurospora sitophila* 299	0.005 — 0.07 γ [c]

[a] Medium 48a, Table XXII with biotin omitted.
[b] Medium 48a, Table XXII.
[c] Medium 48a, Table XII with 3γ of added thiamine per 10 cc.

Stock cultures are carried and inocula prepared as described under choline assay, above.

Procedure.—The appropriate medium (Medium 48a, Table XXII, modified where necessary as indicated above) is prepared at twice the concentration indicated in Table XXII. Five cc. quantities are added to 50 cc. Erlenmeyer flasks, and diluted to 10 cc. by additions of the vitamin-containing samples and water. Flasks are plugged with cotton, sterilized for 10 minutes at 15 lbs. pressure, cooled, and inoculated with one drop of the proper conidial suspension. Incubation periods of three days at 25° are adequate for all of the vitamins.

Measurement of response.—The mycelium was removed from each flask, placed on a filter paper in a Buchner funnel, washed well with distilled water, removed from the filter paper, dried in an oven at 90° for 2 hours, then weighed directly on the pan of an analytical balance to the nearest 0.1 mg.

Comment.—The general procedure has been applied primarily to samples of epidermis during carcinogenesis. Quantitative recoveries of each vitamin added to epidermal extracts were obtained.

V. Methods Utilizing Miscellaneous Microorganisms

1. Available Microorganisms for Use in Bioassays

As indicated in the introductory portion of this chapter, any microorganism which specifically requires a given substance for growth can be used for its determination if proper conditions are developed. Many miscellaneous microorganisms have been thus used for individual vitamins. In this section the use of such microorganisms will be described. It will not be possible to treat the detailed nutritional requirements of each of these microorganisms as has been done for the more generally useful types of assay microorganisms. For the nutritional requirements of these organisms, reference should be made to previously cited reviews (22, 21), and to standard bacteriological reference works.

Oftentimes, the requirement of an organism for a certain vitamin has been proved, and its use for assays suggested, but no application of the method to natural materials has been made. Such procedures will not be considered below.

2. Assay Methods for Individual Vitamins

A. *p*-aminobenzoic acid

(1) Assay Media and Their Development

The composition of basal media for determination of *p*-aminobenzoic acid with miscellaneous microorganisms is shown in Table XXIII. Medium 49 was developed for use with *Clostridium acetobutylicum,* for which biotin and *p*-aminobenzoic acid are the only vitamin requirements (336). Following the demonstration that *p*-aminobenzoic acid, pantothenic acid or its lactone moiety, and nicotinic acid were growth essentials for *Acetobacter suboxydans* (337), Landy and Dicken (338) developed a procedure for use of this organism for *p*-aminobenzoic acid assay. It was later found (339) that the response to *p*-aminobenzoic acid was greatly sensitized if purine bases were added to the basal medium, and the modified medium (No. 50) is given in the table. Still later, it was shown (340) that inclusion of glucose, and Norit-treated peptone and liver concentrates further improved growth of this organism in the presence of excess *p*-aminobenzoic acid (Medium 51). For methods of extracting *p*-aminobenzoic acid from natural materials, reference should be made to p. 414.

(2) Summary of the Assay Methods

a. Method of Lampen and Peterson (249).

Assay organism—*Clostridium acetobutylicum* S9, ATCC No. 9594. A sporulated culture is dried on sterile soil; the spore stock thus obtained remains viable for years.

Basal medium—Medium 49, Table XXIII.

Preparation of unusual ingredients of the basal medium—

(1) Casein hydrolyzate—100 g. of Labco "Vitamin-free" casein are refluxed 20 hr. with 1 l. of 20% HCl. The hydrolyzate is reduced *in vacuo* to a thin syrup, 300 cc. of water added, and the water again removed. This procedure is repeated a second time. The resulting syrup is brought to pH 4.0 with NaOH, and diluted to 1 l.

One hundred cc. of this hydrolyzate are stirred on a steam bath with 2 g. of Norit for 30 min., cooled and filtered. The filtrate, although much lower in *p*-aminobenzoic acid than the original hydrolyzate, is not sufficiently low for use in assays. It is adjusted with H_2SO_4 to pH 2.0, and extracted continuously with ether for 24 hr. The unextracted residue is diluted to 100 cc. and stored until use. One cc. is equivalent to 100 mg. of original casein.

Preparation of inoculum—A loopful of the soil spore stock is added to a medium consisting of glucose, ammonium phosphate, ammonium acetate, and salts, all at one-half the concentrations indicated in Medium 49, and containing 0.5% peptone. After 24-30 hr. incubation (37° C.), 4 drops of this culture are transferred to a tube of inoculum medium (5 cc. of basal medium, 5 mγ of *p*-aminobenzoic acid, and water to make 10 cc.) When 18-24 hr. old, the culture is ready for use as inoculum. One drop is added to each assay tube. Inocula may be subcultured 3 or 4 times for use on successive days before a return is made to the soil stock.

Procedure—Assays are carried out in 19 × 150 mm. test tubes. Approximately 5 mg. of reduced iron are added to each tube; followed by appropriate aliquots up to 5 cc. of standard *p*-aminobenzoic acid solution and sample solutions. The assay range is from 0 to 2.0 mγ of *p*-aminobenzoic acid/10 cc. Contents of all tubes are diluted to 5 cc. with distilled water; 5 cc. of freshly prepared basal medium are then added. The tubes are plugged with cotton, autoclaved at 15 lb. pressure for 15 min., cooled and inoculated with 1 drop of the inoculum. An anaerobic jar is prepared by filling a suitable container ¼ full of oats and adding just enough water to cover the oats. The assay tubes are placed in the jar, on which a glass top is sealed with plasticine. Respira-

TABLE XXIII

Composition of Media for Assay of p-Aminobenzoic with Miscellaneous Microorganisms

Constituent	Medium No. 49. Lampen and Peterson 1944 (249) (*Cl. acetobutylicum*)	50. Landy, Dicken and Streightoff 1942, 1943 (338, 339) (*A. suboxydans*)	51. Cheldelin and Bennett 1945 (340) (*A. suboxydans*)
		Amount/100 cc. of double strength medium	
Casein hydrolyzate, charcoal-treated	0.1 g.	0.6 g.	1.0 g.
Glucose	4.0 g.	—	0.5 g.
Glycerol	—	10 g.	10 g.
Peptone, Norit A-treated	—	—	0.5 g.
Liver concentrate, Norit A-treated	—	—	0.2 g.
Tryptophan	15 mg.	20 mg.	20 mg.
Cystine	20 mg.	15 mg.	15 mg.
Adenine	—	1 mg.	1 mg.
Guanine	—	1 mg.	1 mg.
Xanthine	—	1 mg.	—
Uracil	—	—	1 mg.
Calcium pantothenate	—	20 γ	20 γ
Nicotinic acid	—	20 γ	20 γ
Biotin	0.2 γ	—	—
Inorganic salts			
Solution A [a]	0.8 cc.	1 cc.	1 cc.
Solution B [a]	0.8 cc.	1 cc.	1 cc.
$(NH_4)_2HPO_4$	0.2 g.	—	—
$Na_2S_2O_4.2H_2O$	10 mg.	—	—
Ammonium acetate	0.4 g.	—	—
Reduced iron	[b]	—	—

[a] As in Table IV.

[b] Not added to the basal medium. Added to assay tubes just before sterilization at rate of approximately 5 mg. per tube.

tion of the oats removes the oxygen and establishes a partial pressure of CO_2. The jar is placed in the incubator at 37° C. for 20-24 hr.

Measurement of response—Following incubation, the jar is opened, the tubes removed and shaken thoroughly, the contents transferred to a suitable receptacle, and growth determined turbidimetrically. The reduced iron forms a coherent mass which remains at the bottom of the tube, and does not interfere with turbidity measurements.

Comment—The procedure is illustrative of that used when sporulating, obligately anaerobic organisms are employed for assay purposes. The precision of the assay is stated to be ± 10%. The assay organism seems to be very specific in its response to *p*-aminobenzoic acid; related structures, with the exception of *p*-nitrobenzoic acid, which can be reduced to the vitamin by the organism, have negligible activity (336). Sample preparation, which was studied quite extensively in the original paper (249), has been discussed previously. Attention is called to the conflicting stabilities of *p*-aminobenzoic acid to acid and alkali treatment, as indicated by assay with lactic acid bacteria and *Cl. acetobutylicum* (p. 414). The discrepancy has not been resolved.

The general factors involved in application of *Cl. acetobutylicum* to assay of *p*-aminobenzoic acid have also been studied by Housewright and Koser (341), but no detailed procedure is given.

b. Method of Landy and Dicken (338) as modified by Landy and Streightoff (339).

Assay organism—*Acetobacter suboxydans,* ATCC No. 621. Carried as slant cultures on yeast extract-glycerol-agar (0.5% Bacto-yeast extract, 5% glycerol, 1.5-2.0% agar, pH 6.0), with transfer at monthly intervals. Incubated at 30° C. until growth on the slant is heavy, then held in the refrigerator until the next transfer.

Basal medium—Medium 50, Table XXIII. Initial pH, 6.0 ± 0.1.

Preparation of casein hydrolyzate—A commercially available casein hydrolyzate (General Biochemicals, Chagrin Falls, Ohio) is stated to be sufficiently free of *p*-aminobenzoic acid for use.[54] Other purified casein hydrolyzates, prepared, *e.g.,* as on pp. 364 or 471 are also satisfactory.

Inoculum medium—Five cc. of basal medium, 0.01 γ of *p*-aminobenzoic acid and water to 10 cc. per 50 cc. Erlenmeyer flask. Plugged, sterilized and held until use.

Inoculum—A transfer from the stock culture to a flask of inoculum medium is incubated at 30° C. for 24 hr. Cells are centrifuged out, washed twice in 10 cc. of sterile saline, and finally resuspended in 15 cc. of sterile saline. One drop of this suspension per flask serves as the inoculum.

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[54] In some instances, this has not proved to be the case. It can be freed of interfering amounts of the vitamin by adsorption with 10 per cent of its weight (solids basis) of activated charcoal at pH 2.0-3.0.

Procedure—Five cc. of basal medium and appropriate aliquots (up to 5 cc.) of the standard and sample solutions are placed in 50 cc. Erlenmeyer flasks. The assay range is from 0.5 to 10 mγ of *p*-aminobenzoic acid per flask. All volumes are adjusted to 10 cc. with water, flasks are plugged with cotton and autoclaved at 15 lb. pressure for 15 min., cooled, inoculated with 1 drop of the inoculum suspension, and incubated at 30° C. for approximately 48 hr. For a discussion of methods of preparing samples for assay, see p. 414.

Measurement of response—Turbidimetric, following thorough shaking of cultures. Standards are plotted and results obtained in the usual fashion.

Comment—The method is one of the simplest and most dependable so far suggested for assay of *p*-aminobenzoic acid. A modified medium (No. 51, Table XXIII) which permits somewhat greater growth in response to increments of *p*-aminobenzoic acid (340) has been suggested; no comparisons have been made to determine whether such modification enhances precision of the assay.

With the unmodified medium, recoveries of *p*-aminobenzoic acid added to various test materials range from 90 to 105%. Assay values calculated at different dosage levels of test samples agree satisfactorily. As computed from such data the method appears accurate to about ± 15%. A number of compounds related to *p*-aminobenzoic acid in structure were tested and found to be essentially inactive.

If the purine bases are left out of the basal medium, growth occurs as before with excess *p*-aminobenzoic acid, but the amount of the vitamin required is increased about 5- to 10-fold (339).

c. Method of Cheldelin and Bennett (340). This method represents a modification of, and is essentially identical with, that of Landy *et al.*, described above, except that medium 51, Table XXIII, is used with an incubation period of 60-70 hr. No fuller description is, therefore, necessary. The crude supplements required for the modified medium are prepared in the following manner (342).

(1) Norit A-treated peptone and liver concentrate—Ten g. of liver concentrate (nature unspecified, obtained from Lederle Laboratories, Inc.) or of 1:20 liver powder (Wilson Laboratories) and 25 g. of Bacto-peptone (Difco Laboratories) are dissolved in water, diluted to 500 cc., steamed 10 min., and cooled. The pH is adjusted to 1.5, the solution shaken for 20 min. with 35 g. of Norit A, and filtered. The pH is read-

justed to 1.5 and the charcoal treatment repeated. The filtrate is now adjusted to pH 6.0, steamed, cooled, filtered, and preserved in the refrigerator under toluene. It contains the equivalent of 50 mg. of peptone and 20 mg. of liver concentrate/cc.; 10 cc. of it is used for each 100 cc. of the double strength medium.

(2) Casein hydrolyzate—A Norit-treated, acid-hydrolyzed, vitamin-free casein is prepared in the same manner as described elsewhere (*e.g.*, pp. 364, or 471).

B. BIOTIN

Biotin is required for growth of a great many bacteria, yeasts, and molds. However, aside from those organisms whose use for its assay has already been considered (lactic acid bacteria, yeasts and *Neurospora*), only *Clostridium butylicum* and *Rhizobium trifolii* appear to have been used for determination of biotin in natural materials. The former organism was shown to require biotin as the sole growth factor in 1939 (343); a preliminary report of its use for assay of natural materials appeared in 1940 (344), and details of the method, in 1942 (163). The method is not as convenient as others which have appeared, but will be summarized below as illustrative of the use of a different type of assay organism.

Rhizobium trifolii was shown to require a vitamin-like substance for growth in synthetic media as early as 1934 (345). This was called "coenzyme R" and subsequently was identified as biotin (346, 347). The response of this organism has been adapted for biotin assay by West and Woglom (348), and this method will be treated briefly below.

Ashbya gossypii was used to assay for biotin in preliminary studies by Robbins and Schmidt (349), but the method has not been further elaborated, and will not be considered below.

(1) Method of Lampen, Bahler and Peterson (163)

In details not described fully below, the method is similar to that of Lampen and Peterson, p. 471, which uses a closely related organism for the determination of *p*-aminobenzoic acid.

Assay organism—*Clostridium butylicum* No. 21 (Wisconsin). Carried as a spore stock on sterile soil.

Basal medium—Medium 52, Table XXIV. Initial pH, 6.6-6.7.

Inoculum medium—Basal medium plus 0.25% peptone. Tubed in 26 cc. lots (25 × 200 mm. test tubes) with a small amount of reduced iron in each tube to aid in maintaining anaerobic conditions.

Inoculum—A loopful of spore stock is transferred to a tube of the basal medium, incubated anaerobically for 18-20 hr. at 37° C. Ten cc. of the culture is centrifuged and the cells resuspended in 10 cc. of sterile, freshly-boiled water. Three drops of this suspension are used to inoculate each assay tube.

Procedure—Lipless test-tubes, 25 × 200 mm., and graduated at 36 cc., are used. A small amount of reduced iron is added to each tube. Appropriate aliquots (10 cc. or less) of the standard biotin solution or an extract of the material to be assayed, are placed in the tubes. The assay range is from 0 to about 0.03 γ; 0.01 γ gives half maximum growth. Volumes are made in all cases to 11 cc. with water, then 25 cc. of the basal medium are added. Tubes are plugged, autoclaved 30 min. at 15 lb. pressure, cooled, and inoculated immediately. Incubation is in an anaerobic oat jar at 37° C. for 3 days.

Measurement of response—Turbidimetric. On the Evelyn colorimeter, cultures containing excess biotin read between 15-20.

Comment—The assay medium is extremely simple, but the organism is comparatively inconvenient to handle because of its requirement for anaerobic cultural conditions. Addition of thioglycollate, agar, cysteine or sodium hydrosulfite failed to obviate the necessity for anaerobic incubation. The authors state that "it has become evident that there is considerable variation between duplicates even on pure biotin"; the accuracy claimed is consequently somewhat less (± 20%) than that claimed for most other microbiological methods. A considerable study was made with the method of hydrolytic procedures necessary to release biotin from bound forms which occur in tissues. For a discussion of this problem see p. 388.

(2) Method of West and Woglom (348)

Assay organism—*Rhizobium trifolii* 205. Carried on agar slants of the basal medium plus 1.5% agar and 0.1% Difco yeast extract. Incubated at 28° C.

Basal medium—Medium 53, Table XXIV. Initial pH 6.8.

Inoculum—A light suspension of *Rhizobium trifolii* cells in water is prepared by removing cells from a stock culture of the organism and uniformly suspending in water. Such a suspension should contain 50 to 100 million cells/cc. One loopful of this suspension is used to inoculate each flask.

Procedure—Various dilutions of standard and of extracts to be assayed are measured in amounts from 0.2 cc. to 0.001 cc. into 125 cc. Erlenmeyer flasks. 25 cc. of basal medium are added. After autoclaving (30 min. at 15 lb.) and cooling, each flask is inoculated, then incubated for 5 days at 28° C. The assay range is from 0 to about 1 mγ of biotin in 25 cc. of medium.

Measurement of response—Turbidimetric. Results are determined by comparing the amount of extract necessary to produce half-maximum growth of the test organism with the amount of biotin similarly required. The latter approximates 0.32 mγ/25 cc. of medium.

TABLE XXIV

Composition of Media for Assay of Biotin with Clostridium butylicum and Rhizobium trifolii

Constituent	Medium No. 52. Lampen, Bahler and Peterson, 1942 (163) (*Cl. butylicum*)	Medium No. 53. West and Woglom, 1942 (348) (*Rhiz. trifolii*)
	Amount/100 cc. of basal medium.[a]	
Glucose	2.88 g.	—
Mannitol	—	0.5 g.
Asparagine	0.144 g.	—
Inorganic salts		
Solution A[b]	0.64 cc.	—
Solution B[b]	0.64 cc.	—
K_2HPO_4	—	50 mg.
KNO_3	—	20 mg.
$MgSO_4.7H_2O$	—	20 mg.
NaCl	—	10 mg.
$CaSO_4$	—	10 mg.
$FeCl_3$	—	1 mg.
Thiamine	—	10 γ
β-Alanine	—	10 γ

[a] Medium 52 is 1.42 times its final concentration; medium 53 is at its final concentration. The latter medium is boiled and filtered hot before use.

[b] As in Table IV.

Comment—Few or none of the usual criteria applied to determine the accuracy of microbiological assays in general have been applied in this case. The method was applied to a variety of cancer and normal tissues by its authors, but has not been used elsewhere. It is not possible, therefore, to give any estimate of its validity as compared with other methods.

C. NICOTINIC ACID

(1) Assay Media and Their Development

Strains of *Proteus vulgaris* and of *Shigella paradysenteriae* have been suggested for determination of nicotinic acid or its amide in natural materials; indeed, proponents of these methods were among the first to suggest the utilization of microorganisms for the determination of vitamins in natural materials.

Fildes (350) showed, in 1938, that nicotinic acid was the only growth factor required by *Proteus vulgaris*. Medium 54, Table XXV, is a modification of his original medium, developed by Lwoff and Querido (351) for use with this organism and natural materials. Koser, Dorfman and Saunders (352), also in 1938, showed that dysentery bacilli required nicotinic acid for growth. This finding was applied to assay of nicotinamide by Fraser, Topping and Sebrell (353), using *Shigella paradysenteriae* (Sonne), and by Dorfman *et al.* (354, 355) who used *Shigella paradysenteriae* (Flexner) as test organism. The former procedure was modified by Isbell *et al.* (356); the modified medium (No. 55, Table XXV) is similar to that of Lwoff and Querido. The medium of Dorfman *et al.* is also given (No. 56).

(2) The Assay Methods

a. Method of Lwoff and Querido (351).[55]

Assay organism—*Proteus vulgaris* X19. Carried by monthly transfer on peptone-agar slants. Incubated over night at 37° C., then held at laboratory temperatures until next transfer.

Basal medium—Medium 54, Table XXV, initial pH adjusted with NaOH to 7.5. The medium is prepared and sterilized without the glucose, or the ferric citrate, which are sterilized in separate solutions and added aseptically just before use.

Inoculum—The culture must be adapted to the synthetic medium by transferring a comparatively large number of cells to 5 cc. of the basal medium supplemented with 10 γ of nicotinic acid. Daily transfers of 5 drops of the previous culture into 5 cc. of the supplemented medium are made until the culture grows heavily in the synthetic medium. For inoculating assay flasks, a culture low in nicotinic acid is prepared by transferring a drop of the "adapted" culture to 5 cc. of medium supplemented with only 1 γ of nicotinic acid. Daily serial transfers are made from this into (a) the same supplemented (1 γ nicotinic acid/5 cc.) medium, and (b) an unsupplemented medium. When the culture in series (b) fails to grow, or shows negligible growth, the cultures in series (a) can be used as inoculum. This may require 2 or 3 transfers. All cultures are incubated at 28° C.

Procedure—A sterile medium is prepared by mixing various ingredients in the following proportions: 95 cc. of the basal medium (sterile, without glucose or iron citrate), 1 cc. of sterile 30% glucose solution and 0.5 cc. of sterile ferric citrate solution (1 g. in 1000 cc. of water). This

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[55] The details of this method, which are not well described in the original publications, have been treated in full by Morel (357).

TABLE XXV

Composition of Media for Assay of Nicotinic Acid with Miscellaneous Microorganisms

	Medium No.		
Constituent	54. Lwoff and Querido 1938-9 (351) (*Proteus vulgaris*)	55. Isbell, Woolley, Butler and Sebrell 1941 (356) (*Shigella paradysenteriae*)	56. Dorman, Koser, Horwitt, Berkman and Saunders 1940 (354, 355) (*Shigella paradysenteriae*)
	Amount/100 cc. of basal medium.[a]		
Glucose	0.3 g.	1.0 g.	0.5 g.[b]
KH_2PO_4	0.45 g.	1.0 g.	—
K_2HPO_4	—	—	0.1 g.
$(NH_4)_2SO_4$	75 mg.	150 mg.	—
KCl	50 mg.	100 mg.	—
$MgSO_4.7H_2O$	5 mg.	10 mg.	10 mg.
Fe citrate	0.5 mg.	—	—
$Fe(NH_4)_2(SO_4)_2.6H_2O$	—	19.6 mg.	—
Thiamine chloride	—	2 mg.	—
Amino acids	—	—	0.275 g.[c]

[a] The ingredients of Medium 54 are sufficient for 96.5 rather than 100 cc. of medium; the volume is made to 100 cc. by incorporation of the sample. Medium 55 is double strength, and is, therefore, much more similar to medium 54 than it appears. Medium 56 is 1.1 times its final concentration.

[b] Sterile glucose solution is added to the previously autoclaved solution containing the other ingredients of the medium, to make a final glucose concentration of 0.5%.

[c] L-glutamic acid 50 mg., L-alanine 50 mg., glycine 20 mg., L-lysine.2HCl 20 mg., L-tryptophan 20 mg., L-histidine 20 mg., DL-valine 10 mg., L-leucine 10 mg., DL-phenylalanine 10 mg., L-cystine 10 mg., L-proline 10 mg., L-hydroxyproline 10 mg., DL-methionine 10 mg., L-arginine.HCl 10 mg., L-tyrosine 5 mg.

is dispensed in 9 cc. portions to sterile 100 cc. Erlenmeyer flasks. One cc. of a sterile supplement (the nicotinic acid standard or sample) is added aseptically. Each extract (sample and standard) is set up at two different concentrations, with duplicate flasks at each level. The assay range is from 0.01 to 0.12 γ of nicotinic acid/10 cc. of medium. The flasks are inoculated with 2 drops of an inoculum culture (24 hr. old) and incubated at 28° C. for 36 hr. They may be called flasks "D." Controls are also prepared as follows and incubated: (a) Unsupplemented, inoculated, medium, (b) uninoculated medium, and (c) medium + sample, uninoculated.

Measurement of response and calculation of results—After the 36 hr. incubation period, each flask is shaken (no air bubbles) and the optical density determined. The optical density due to the added nicotinic acid will be "d;" and is given by the formula $d = D - (a + c - b)$, where

D, *a*, *c*, and *b* represent the optical densities found for the corresponding flasks, as measured in the electrophotometer of P. Meunier.[56] The result is obtained by reference to a standard curve relating "*d*" to concentrations of nicotinic acid. Since such a curve is perfectly linear, the curve may be dispensed with and the result found by calculation, if desired.

Comment—The unique feature of this test lies in the fact that, when properly carried out, standard curves are perfectly superimposable from one day to the next, and need, therefore, be run only occasionally. With natural samples, however, deviations from linearity with increased amounts of sample may occur, showing that interfering substances may be present. The amount of growth obtained in response to a given quantity of nicotinic acid depends upon the surface to volume ratio. For best results, flasks must be used as the assay vessel.

It is important that cultures be maintained at 28° C., since at higher temperatures, specifically 37° C., selection of an organism requiring larger amounts of nicotinic acid occurs. The assay has been rather exhaustively investigated from the standpoint of the effect of pure compounds, but much less thoroughly studied with respect to its application to natural materials (357). The accuracy is stated to be ± 10% or greater. Nicotinic acid and nicotinamide are equally active; hydrolysis of the coenzymes is necessary before full activity is shown. Toward other compounds the specificity of the test organism is similar to that of animals (357).

As originally described, the assay would be tedious to carry out; appropriate modifications might make of it a simple and useful method. Such a modification has been recently described by Grossowicz and Sherstinsky (355a) and is given in summary form below.

b. Method of Grossowicz and Sherstinsky (358a).

Assay organism—*Proteus vulgaris* X19. Carried by monthly transfer on peptone-agar slants, with incubation at 30° for 24 hours. Slant cultures are stored in the refrigerator between transfers.

Inoculum—A small amount of growth is removed with a wire loop from the surface of a 24 hour agar slant of the test organism. Care should be taken to avoid direct contact with the agar. The loopful of *Proteus* is suspended in enough sterile 0.9% NaCl solution to yield a barely turbid suspension; 1 cc. of this is added to 100 cc. of saline. 2

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[56] With this instrument, if the optical densities are plotted as ordinates against dilutions of a given turbid culture, a straight line is obtained over the range used. This is true also for such photoelectric colorimeters as the Evelyn used in this country, provided the optical densities do not surpass about 0.4. This is equivalent to saying that below this optical density, turbid bacterial suspensions obey Beer's law.

drops (0.1 cc.) of this light suspension is used to inoculate each assay tube.

Preparation of hydrolyzed casein for basal medium—50 g. of casein is mixed with 250 cc. of 25% H_2SO_4 and autoclaved for 10 hours at 15 lbs. pressure. Sulfuric acid is then removed with baryta. Excess Ba^{++} is carefully removed with H_2SO_4 (pH 4-5). The hydrolysate is stirred with 2% of norite for 30 minutes; then filtered. If the casein is of technical grade, this treatment should be repeated to assure complete removal of nicotinic acid. If the hydrolysate permits significant growth in the basal medium in the absence of nicotinic acid, it is not sufficiently free of the vitamin. When the nicotinic acid has been completely removed, the hydrolysate is adjusted to pH 7.0 with conc. NaOH, then diluted or concentrated to give a final concentration of 10%.

Basal Medium and Procedure—The medium is a modification of that of Lwoff and Querido (No. 54, Table XXII) but with some alterations in salt content and the addition of casein hydrolysate. It is prepared as follows:

Mineral base medium: 0.75 g. $(NH_4)_2SO_4$, 4.5 g. KH_2PO_4, 0.5 g. KCl and 1.0 g. $NaNO_3$ are dissolved in 750 cc. distilled water, adjusted to pH 7.4 with 2 to 2.5 cc. of 10 N NaOH, then transferred in 7.5 cc. quantities to 16 x 150 mm. test-tubes, which are plugged with cotton and autoclaved at 15 lbs. pressure for 30 minutes. To each tube of this medium one then adds aseptically the indicated amounts of the following separately sterilized solutions: (a) 30% glucose, 0.2 cc., (b) 0.1% ferric citrate, 0.1 cc., (c) 0.5% $MgSO_4$ 0.1 cc., (d) 10% hydrolyzed casein 0.1 cc.

Alternatively, solutions (a), (b), (c) and (d) can be mixed in the proportions indicated, sterilized by Seitz filtration, and 0.5 cc. of the sterile filtrate added aseptically to each tube of the mineral base medium.

To the 8 cc. of sterile medium thus obtained, graded amounts of the standard nicotinic acid solution (sterile) or the sample solution (sterile) are added aseptically, and the volumes adjusted to 10 cc. with sterile distilled water. The assay range is from 0 to 0.1 γ of nicotinic acid. Each tube is then inoculated with one drop of the inoculum suspension, then incubated in a slanted position (10° to 15°, to supply a large surface for oxygen transfer to the aerobic test organism) for 40 hours at 30° C.

Measurement of response—Turbidimetric.

Comment—The specificity is similar to that shown by the same test organism in the method of Lwoff and Querido. The present medium is stated to permit much heavier and more rapid growth than the older medium. Standard curves are not linear, and must be run with each assay. Values closely approximating those found by assay with *L. arabi-*

nosus by the method of Snell and Wright (48) were found for a series of samples.

The chief advantage claimed for the method over those employing *L. arabinosus* is the increased sensitivity: the assay range is between 0.01 and 0.1 γ of nicotinic acid instead of between 0.08 and 0.3 γ, as in acidimetric methods utilizing *L. arabinosus.* It should be pointed out, however, that if the latter organism were used in a turbidimetric (rather than an acidimetric) assay, and growth were restricted to the same levels obtainable with *Proteus* (maximum growth, at 55% light transmission), then the two organisms would show very similar sensitivities to nicotinic acid.

The extensive aseptic manipulations required in the present procedure are inconvenient, and could probably be avoided by appropriate study and modification of the procedure.

b. Method of Isbell, Woolley, Butler and Sebrell (356).

c. Method of Dorfman, Koser, Horwitt, Berkman and Saunders (354, the basal medium. Tubes are plugged and sterilized at 15 lb. for 10 min. Sterile solutions of standard calcium pantothenate and sample dilutions are added in volumes of 0.1 cc. The assay range is from 1-5 mγ of pantothenate/10 cc. Tubes are inoculated and incubated for 24 hr.

c. Method of Isbell, Woolley, Butler and Sebrell (356).

Assay organism—*Shigella paradysenteriae* (Sonne), No. 741 of the National Institute of Health. Carried by bimonthly transfer on beef infusion agar slants, with incubation at 31° C.

Basal medium—Medium 55, Table XXV. Initial pH 7.4-7.6. Becomes turbid on standing, and should, therefore, be prepared fresh on the day of assay by dilution of a concentrated stock solution containing all the ingredients except glucose and ferrous ammonium sulfate.

Inoculum—24 hr. prior to assay, a fresh slant of the test organism is prepared. Growth is removed from it with a loop, and uniformly suspended in sterile saline to give a barely visible suspension. One drop of this is used for each assay tube.

Procedure—Assays are carried out in 20 × 175 mm. test-tubes suitable for direct use in the photoelectric photometer. Appropriate aliquots of the *nicotinamide* standard or sample (up to 5 cc.) are added, then 5 cc. of the basal medium, followed, where necessary, with water to bring the total volume to 10 cc. 0, 0.05, 0.10, 0.15, 0.20 and 0.25 γ of nicotinamide are suitable amounts to establish the standard curve. Tubes are plugged with cotton, autoclaved 15 min. at 15 lb. pressure, cooled, inoculated, and incubated for 16-22 hr. at 31° C.

Measurement of response—Turbidimetric.

Comment—Nicotinic acid is less active than nicotinamide, so that the results obtained, which are expressed in "equivalents to nicotinamide" represent the nicotinamide plus an indeterminate fraction of the free nicotinic acid, and other active combinations of nicotinic acid present. This limits rather severely the utility of the method. Dorfman *et al.* (354, 355) found that the activity of nicotinic acid relative to that of nicotinamide was increased by using longer incubation periods.

Values obtained with the method on natural materials are reproducible to ± 10%. Recoveries of added nicotinamide are also obtained to within ± 10%.

Since nicotinamide, rather than nicotinic acid, is the active substance, and since response to nicotinic acid tends to be slow and irregular, extracts cannot be hydrolyzed preliminary to assay, as recommended on p. 360. No general extraction procedures have been recommended by the authors of the method; some materials are assayed directly, others are heated before assay. With some materials (*e.g.*, urine) heating increases the magnitude of the values observed, perhaps because of breakdown of coenzymes to a utilizable form (358).

d. Method of Dorfman, Koser, Horwitt, Berkman and Saunders (354, 355). The method is similar to that of Isbell *et al.*, described above, but employs a different strain of the same organism (*Shigella paradysenteriae* Flexner), and a different basal medium (Medium 56, Table XXV). Growth is determined by titration of the acid produced after an incubation period of 4 days at 37° C. The maximum acid thus produced is equivalent to approximately 2.6 cc. of 0.01 *N* NaOH. The procedure has been applied only to blood, and will not be described in greater detail.

D. PANTOTHENIC ACID

(1) *The Assay Methods*

Only a single method, other than those employing lactic acid bacteria and yeasts, has been suggested for pantothenic acid. This procedure (359) utilizes as test organism *Proteus morganii*, which requires pantothenic and nicotinic acids for growth on a mineral salts-glucose medium (360). Growth on such a synthetic medium is improved considerably by the addition of extracts of crude materials; for assay purposes it is, therefore, supplemented with a pantothenic acid-free preparation of proteose-peptone. The composition of the basal medium is given in Table XXVI.

a. Method of Pelczer and Porter (344).

Assay organism—*Proteus morganii* 21, ATCC No. 8091. Carried as slant cultures on nutrient agar, with biweekly transfer.

Basal medium—Medium 57, Table XXVI.

Preparation of alkali-treated proteose peptone for the basal medium—A 10% solution of proteose-peptone in 1 *N* NaOH is autoclaved at 15 lb. pressure for 1 hr., cooled, neutralized with glacial acetic acid and filtered to remove any precipitate that forms. The neutral solution is reautoclaved at 15 lb. for 10 min., and stored until use. The alkali treatment frees the peptone of pantothenic acid.

Inoculum—A trace of growth from a 24 hr. slant culture is transferred with a wire needle to 10 cc. of the basal medium containing 5 mγ of calcium pantothenate. After 24 hr. incubation, 1 drop of this culture is transferred to another 10 cc. tube of the same medium. After 24 hr., one drop of this second subculture is used to inoculate each assay tube. Temperature of incubation is not specified, but presumably 37° C.

Procedure—Assays are carried out in test tubes containing 10 cc. of the basal medium. Sterile solutions of standard calcium pantothenate and sample dilutions are added in volumes of 0.1 cc. The assay range is from 1-5 mγ of pantothenate/10 cc. Tubes are plugged and sterilized at 15 lb. for 10 min. Tubes are inoculated and incubated for 24 hr.

Measurement of response—Turbidimetric.

Comment—Reported recoveries of added pantothenate varied from 87 to 108%. In most cases values calculated from different dilutions of test substance were constant. The method has not been widely used; little can be said, therefore, concerning its general applicability.

TABLE XXVI

Composition of Medium for Determination of Pantothenic Acid with Proteus morganii

Medium No.

57. Pelczar and Porter, 1941 (359)

Constituent	Amount/100 cc. of basal medium
KH_2PO_4	0.45 g.
$(NH_4)_2SO_4$	50 mg.
NH_4Cl	50 mg.
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.57 mg.
$MgSO_4.7H_2O$	4 mg.
Glucose	0.50 g.
Nicotinic acid	123 γ
Cystine	160 γ
Proteose-peptone, alkali-treated	0.10 g.

The assay organism is more sensitive to pantothenic acid than are the lactic acid bacteria thus far used for assay, and this should be advantageous in some instances. Some differences in specificity of response to combined forms of pantothenic acid appear to exist between *Proteus morganii* and *Lactobacillus casei.* Use of the former organism for assay of blood yields values of 0.03 to 0.10 γ/cc. (361); *L. casei* gives higher values, ranging from 0.19 to 0.32 γ/cc. (362). Wright found that, if unheated blood or plasma were assayed with *L. casei,* values were obtained which checked the *Proteus* assay; after heating, the higher values were obtained with *L. casei* but not with *Proteus morganii* (362). This interesting finding has not been further explained.

E. THIAMINE

(1) Assay Media and Their Development

Thiamine or its split products are required by perhaps more microorganisms than any other single vitamin which has been investigated. Despite this fact, few microbiological methods, other than those already treated, have been suggested for its determination. The methods considered below are those using *Phycomyces blakesleeanus* and *Staphylococcus aureus* as test organisms. The *Phycomyces* method is based upon the fundamental work of Schopfer (review, 363), who showed in 1935 that pure thiamine was required for growth of this organism on a synthetic medium (364). Its use for thiamine determinations was suggested following an investigation of its utility for this purpose in 1937 (365); the method thus represents perhaps the earliest application of microorganisms to bioassay of a vitamin in crude natural materials.

The effect of environmental conditions on response of *Phycomyces* to thiamine has been investigated rather extensively. The original basal medium (No. 58, Table XXVII) contained 0.1% asparagine as nitrogen source. Increasing this amount of asparagine markedly increases the amount of growth obtained per unit of added thiamine (366, 367, 368), and some natural samples contain sufficient asparagine (or other utilizable nitrogen source) to give the effect (367). For assay, it is advisable, therefore, to use higher levels of asparagine (*cf.* Medium No. 59, Table XXVII). Hypoxanthine increases the response to thiamine over short periods of time, but does not alter the final amount of growth obtained if a prolonged incubation period is used (369, 363). The amount of growth obtained per unit of added thiamine varies markedly with the temperature, increasing as the temperature is decreased from 25° to 10° C. (368, 370). However, at any given temperature growth is proportional to thiamine concentration, so that this variation does not

TABLE XXVII

Composition of Basal Media for Assay of Thiamine with Phycomyces blakesleeanus and with Staphylococcus aureus

Constituent	Medium No. 58. Schopfer, 1935 (376) (*Phyc. blakesleeanus*)	59. Hamner, Stewart and Matrone, 1943 (373) (*Phyc. blakesleeanus*)	60. West and Wilson, 1938 (374) (*Staph. aureus*)
		Amount/100 cc. of basal medium.[a]	
Glucose	3 g.	5. g.	0.6 g.
Hydrolyzed casein	—	—	0.8 g.
Asparagine	100 mg.	500 mg.	—
$MgSO_4.7H_2O$	50 mg.	50 mg.	—
KH_2PO_4	150 mg.	150 mg.	—
K_2HPO_4	—	—	1.0 g.
Ferric citrate (0.1 per cent solution)	—	0.022 cc.	—
Trace element solution	—	0.01 cc.[b]	—
Cysteine. HCl	—	—	4.0 mg.
Nicotinic acid	—	—	40 γ

[a] Media 58 and 59 are at essentially their final concentration; medium 60 is twice its final concentration.

[b] Each liter of this solution contains the following salts: $ZnSO_4$, 0.245 g.; $CuSO_4$, 0.0502 g.; $MnSO_4$, 0.0275 g.; H_3BO_3, 0.0572 g.; $(NH_4)_2$ MoO_4, 0.0327 g.

argue against use of the assay. Similarly, growth per unit of thiamine increases as the surface area of the nutrient solution is increased (stationary incubation); again, under constant conditions of assay, this factor is eliminated (368).

Of the various procedures which have been described for use with *Phycomyces* (367, 371, 372, 373), all of which are very similar to the original procedures of Schopfer (365), that of Hamner *et al.* (373) will be described below. The composition of the medium used (No. 59) is compared with that of the original medium (No. 58) in Table XXVII. For a review dealing with the method, see (363).

The method of West and Wilson (374) grew out of the observation of Knight (9, 375) that thiamine was required for growth of *Staphylococcus aureus* on a purified medium. The basal medium used (No. 60, Table XXVII) is a modification of the earlier medium used by Knight.

(2) The Assay Methods

a. Method of Hamner, Stewart and Matrone (373).

Assay organism—*Phycomyces blakesleeanus.* Stock cultures carried on agar slants of medium containing 0.1 g. Witte's peptone, 1 γ of thiamine and 2 g. of agar/100 cc. Transferred at unspecified intervals at least 3 weeks apart. Incubated at 24° C.

Basal medium—Medium 59, Table XXVII.

Inoculum—A spore suspension is prepared by shaking the spores from a tube of the stock culture (2-3 weeks old) in 25 cc. of sterile distilled water. Each assay flask is inoculated with one drop of this suspension.

Procedure—Twenty-five cc. of basal medium are added to the required number of 125 cc. Erlenmeyer flasks. Known amounts (0.1-0.6 γ; increments of 0.1 γ) of thiamine are added to one series of flasks to establish a standard curve, aliquots of sample to supply between 0.1-0.6 γ of thiamine are added to another series of flasks. Flasks are plugged with cotton, sterilized in the autoclave at 15 lb. for 15 min., cooled and inoculated. Incubation is at 24° C. for 2 weeks.[57]

Measurement of response—The mycelial mat present in each flask is transferred to a Büchner funnel lined with hard filter paper, and washed thoroughly with distilled water, rolled into a ball with the fingers, and dried in a suitable container for 24-48 hr. at 100° C. After cooling and allowing to come to equilibrium with the moisture of the air, they are weighed. A standard curve is constructed from which assay values are taken in the usual fashion.

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[57] Shorter periods (as low as 6 days) have been used; the authors find more uniform results with the longer assay period.

Comment—The test organism responds to cocarboxylase and to equimolecular mixtures of the thiazole and pyrimidine component of thiamine in the same fashion as to thiamine itself (363); where the latter two substances occur naturally, as in some heated products, high results can be expected. However, in many products, the mixture apparently does not occur in interfering amounts. Hamner *et al.* (373) obtained good agreement between the fungus growth method and the thiochrome assay in a number of different plant products; on meats, however, the mold growth procedure gave results which were consistently higher by 30-100%. These authors point out that corrections for the preexistent pyrimidine plus thiazole could presumably be made by a sulfite-cleavage procedure similar to that employed by Schultz *et al.* in the yeast fermentation method (310).

In the presence of limiting amounts of thiamine, the dry matter produced by *Phycomyces* is materally increased by an excess of the thiazole moiety of thiamine (377, 216), although thiazole by itself is completely inactive. This interesting phenomenon apparently results from presence of an enzyme which destroys a portion of the intact thiamine by disrupting the thiazole moiety, but leaves the pyrimidine portion unharmed. With added thiazole, the pyrimidine portion can be resynthesized to thiamine, which is then reutilized (370, 377).

These complications make it quite obvious that the *Phycomyces* method, like most other methods, can be applied validly in some instances, but should be thoroughly investigated for suitability before application to previously uninvestigated products.

Many fungi are known which require the intact thiamine molecule. It would appear possible, at present, therefore, to develop a more specific assay for this vitamin, based on fungal growth.

b. Method of West and Wilson (374).

Assay organism—*Staphylococcus aureus.* Carried on nutrient agar. Details of strain and frequency of transfer unspecified.

Basal medium—Medium 60, Table XXVII. Initial pH 7.0.

Inoculum—A trace of growth from a nutrient agar culture is suspended in 10 cc. of water (*ca.* 100,000 bacteria/cc.). One drop is used to inoculate each assay tube.

Procedure—Five cc. quantities of the basal medium are tubed and sufficient distilled water added so that, together with the addition of the test material, the final volume will be 10 cc. The tubes are plugged and autoclaved 1 hr. at 15 lb. pressure. After autoclaving and cooling, the vitamin B_1 and samples are added aseptically. The assay range is from 0 to 5 mγ/10 cc. of final medium. Incubation is at 37° C. for 36 hr.

Measurement of response—Turbidimetric.

Comment—The method has not been widely used or thoroughly tested. Under some conditions, the test organism is pathogenic for man, and must, therefore, be handled with rigid bacteriological technique.

Of 30 strains of *Staph. aureus* investigated, Knight (378) found none which required the intact thiamine molecule; all required both the pyrimidine and thiazole portions to replace it. In this respect, the specificity of this test is very similar to that of the *Phycomyces* test, considered above.

VI. Index to Methods

1. Vitamin to Test Organism

In Table XXVIII, the various vitamins for which microbiological methods are available are listed, together with the test organisms which have been used, references, and the pages on which the method has been summarized. The table does not include the great number of additional organisms which are known to require these vitamins, and which, therefore, might be used for their determination, but only organisms which have actually been used for assay of natural materials.

2. Test Organism to Vitamin

In Table XXIX, various test organisms which have been used for vitamin assay are listed, together with those vitamins which have been determined with them. As in Table XXVIII, the great number of organisms which might be used for assay of individual vitamins has not been listed. Similarly, many of the organisms listed are known to require vitamins other than those listed, and might be applied to their determination with great ease; such application has not been tested, however, and these vitamins are not listed for this reason.

3. Preferred Methods of Assay

To the novice, choice of a method of assay is often difficult because of the large number of available procedures. A list of "preferred" methods is given in Table XXX. It must be admitted that any such list of methods will vary with the experience and personal preferences (or prejudices) of its author. The list below is no exception. In compiling it, the following points have been given most emphasis:

(a) Widely used and tested procedures are preferred over modifica-

TABLE XXVIII

An Index to Microbiological Methods for the Various Vitamins Considered in this Chapter

Vitamin	Test organism	References	Assay described on pages
p-Aminobenzoic acid	*Acetobacter suboxydans*	338, 339, 340	473, 474
	Clostridium acetobutylicum	249	471
	Lactobacillus arabinosus	40	416
	Leuconostoc mesenteroides	41	417
	Neurospora crassa (mutant)	248, 324a	459, 468
Biotin	*Clostridium butylicum*	163	475
	Lactobacillus arabinosus	164, 181	393, 418
	Lactobacillus casei	49, 179, 180	391, 418
	Neurospora crassa	334, 324a	467, 468
	Rhizobium trifolii	348	476
	Saccharomyces cerevisiae	282, 277, 210, 162	425, 427, 428
	Streptococcus faecalis	181	393, 421
Choline	*Neurospora crassa* (mutant)	334, 326, 324a	464, 467, 468
Folic acid	*Lactobacillus casei*	49, 51, 67	401, 404, 418
	Streptococcus faecalis	207, 211, 67	400, 401, 421
Inositol	*Neurospora crassa* (mutant)	335, 324a	468
	Saccharomyces carlsbergensis	282	445
	Saccharomyces cerevisiae	293, 290	441, 444
Nicotinic acid	*Lactobacillus arabinosus*	48, 50, 121, 118	364-69, 418
	Lactobacillus casei	49, 51	418
	Leuconostoc mesenteroides	37, 136	370
	Shigella paradysenteriae	354, 355, 356	482, 483
	Proteus vulgaris	351, 358a	478, 480
	Torula cremoris	295	446
Pantothenic acid	*Lactobacillus arabinosus*	156, 157	385, 386, 418
	Lactobacillus casei	47, 45, 53, 154, 49, 51	382, 383, 384, 418
	Proteus morganii	359	484
	Saccharomyces carlsbergensis	30	430
	Saccharomyces cerevisiae	283	430
Riboflavin	*Lactobacillus casei*	89, 27, 90, 46, 75, 49, 51	340-54, 354, 355, 418, 357
	Leuconostoc mesenteroides	109	355
Thiamine	*Endomyces vernalis*	298, 299	451
	Lactobacillus fermenti	35	376
	Phycomyces blakesleeanus	376, 373	487
	Saccharomyces cerevisiae	297, 298, 299, 310	449, 452-57
	Staphylococcus aureus	374	488
	Streptococcus salivarius	68	373

TABLE XXVIII (continued)

Vitamin	Test organism	References	Assay described on pages
Vitamin B_6	*Lactobacillus casei*	49, 225, 227	411-12
	Neurospora sitophila (mutant)	223, 324a	462, 468
	Saccharomyces carlsbergensis	221	438
	Saccharomyces cerevisiae	224, 284	436, 437
	Streptococcus faecalis	225, 235	412-13

TABLE XXIX

An Index to Microorganisms Used for Assay of Vitamins in Natural Materials[a]

Test organism	Recommended for determination of
BACTERIA	
Acetobacter suboxydans	*p*-Aminobenzoic acid
Clostridium acetobutylicum	*p*-Aminobenzoic acid
Clostridium butylicum	Biotin
Lactobacillus arabinosus	*p*-Aminobenzoic acid, biotin, nicotinic acid, pantothenic acid
Lactobacillus casei	Biotin, folic acid, nicotinic acid, pantothenic acid, riboflavin, vitamin B_6 (pyridoxal)
Lactobacillus fermenti	Thiamine
Leuconostoc mesenteroides	*p*-Aminobenzoic acid, nicotinic acid, riboflavin
Proteus morganii	Pantothenic acid
Proteus vulgaris	Nicotinic acid
Rhizobium trifolii	Biotin
Shigella paradysenteriae	Nicotinamide
Staphylococcus aureus	Thiamine
Streptococcus faecalis	Biotin, folic acid, vitamin B_6, (pyridoxal and pyridoxamine)
Streptococcus salivarius	Thiamine
MOLDS	
Neurospora crassa (mutants)	*p*-Aminobenzoic acid, biotin, choline, inositol
Neurospora sitophila (mutants)	Vitamin B_6 (pyridoxal + pyridoxamine + pyridoxine)
Phycomyces blakesleeanus	Thiamine
YEASTS *Endomyces vernalis*	Thiamine
Saccharomyces carlsbergensis	Biotin, inositol, pantothenic acid, vitamin B_6 (pyridoxal + pyridoxamine + pyridoxine)
Saccharomyces cerevisiae	Biotin, inositol, pantothenic acid, thiamin, vitamin B_6 (pyridoxal + pyridoxamine + pyridoxine)
Torula cremoris	Nicotinic acid

[a] For references and page numbers of treatment in this chapter, refer back to Table XXVIII.

tions which, though they may prove on thorough examination to be superior, have not been subjected to the test of widespread use.

(b) Since the most widely used procedures employ lactic acid bacteria, and all assays using these organisms are very similar, assays using these organisms are preferred in the absence of clearly superior methods.

(c) From the standpoint of simplicity and minimum expenditure of time, assays employing yeast are usually preferable to those using lactic acid bacteria; the latter, in turn, are preferable to those using *Neurospora* or other fungi.

It must also be recognized that the choice of method will depend upon the particular application to be made of it. No consideration of this type can be made in Table XXX.

TABLE XXX

Preferred Methods of Assay

Vitamin	Test Organism	Reference	Assay described on pages
p-Aminobenzoic acid	*Neurospora crassa* (mutant)	248, 324a	468
Biotin	*Lactobacillus arabinosus*	164	393
	Saccharomyces cerevisiae	282, 277	428-29
Choline	*Neurospora crassa* (mutant)	326	464-65
Folic acid	*Lactobacillus casei*	51	404-5
	Streptococcus faecalis	67	401-4
Inositol	*Saccharomyces carlsbergensis*	282	445
Nicotinic acid	*Lactobacillus arabinosus*	50, 48	364-69
Pantothenic acid	*Lactobacillus arabinosus*	156	385
	Saccharomyces cerevisiae	30	430
Riboflavin	*Lactobacillus casei*	46, 27	340-54
Thiamine	*Lactobacillus fermenti*	35	376
	Saccharomyces cerevisiae	310	452-57
Vitamin B_6			
Total	*Saccharomyces carlsbergensis*	221	438
Pyridoxamine + Pyridoxal	*Streptococcus faecalis*	235	412-13
Pyridoxal	*Lactobacillus casei*	227	411-12

VII. Concluding Remarks

The need for accurate and sensitive methods for bioassay of vitamins is attested by the large number of microbiological methods which have been developed, most of which have been summarized above. Consideration of these methods emphasizes the complexity of even the simplest of them. An infinite number of variations in technique, media, *etc.*, is possible. The natural tendency to attempt improvement in the methods has led, in some cases, to a fairly large number of modified procedures. Apart from extraction procedures, however, there is little evidence that many of these modified procedures represent actual improvements in the original methods, and such evidence (apart from qualitative expressions of subjective impressions) is generally lacking in the published modifications, even where these do, in fact, represent definite improvements.

Quite generally, more investigation of the limits of precision of microbiological assays would be very informative. While premature standardization of techniques should be avoided, comparative data might well be obtained to prove the superiority of recommended variations from established practice, unless such variation can be justified on grounds of increased speed or convenience of assay. Similarly, with respect to modified media, it should be emphasized that improved growth on a modified medium is not necessarily accompanied by increased precision or convenience, provided the growth responses on the original medium lie in a range which is easily measurable. Other data are necessary to prove the superiority of such modified media for assay purposes.

It has become evident that many of the weaknesses of microbiological methods lie in inadequate extraction procedures for the vitamins. In many cases, this is a problem which is quite separate from that of the assay *per se*, and should be investigated separately. It is also true, of course, that assay procedures which are inadequate when applied to crude extracts may become very satisfactory if used with an extraction procedure which eliminates interfering materials.

While the application of microorganisms to the determination of vitamins only has been considered above, it should be emphasized that similar procedures are useful in a much wider field. The synthetic capacities of microorganisms vary over a much wider range than those of animals; consequently, products may be required by microorganisms at a stage more or less complex than those required by animals. With thiamine, for example, organisms are known which require only the pyrimidine portion of the molecule, or only the thiazole portion. Some require both halves, others the whole molecule. Assays can be developed which are specific for any stage, and which can be used to study the metabolism of thiamine in plants or animals (*cf.* 377, 379, 21). On the more complex side lie certain strains of the gonococcus, which require cocarboxylase for growth, but cannot utilize thiamine (380). With other vitamins, organisms are known which require, and in some cases have been used to determine, the β-alanine (381) and pantoic acid (342) portions of pantothenic acid, coenzymes I and II (*e.g.*, 382), *etc.* Consideration of such procedures lies outside the scope of this chapter, but their utility in the field of vitamin chemistry is obvious.

Finally, the role microorganisms are playing in the elucidation of the nutritional requirements of animals should be reemphasized. Several of the vitamins were first discovered by their effects on microorganisms; these have also served as test organisms to guide efforts leading to the isolation and synthesis of several vitamins. At present, several unidentified "growth factors" for microorganisms have been reported (*e.g.*, 22, 184, 383, 384, 385, 386, 387, 388); on the basis of past history, it is not unreasonable to expect that some of these may prove of great importance in animal nutrition as well.

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The Use of Optical Instruments in Vitamin Analysis

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All physicochemical methods of vitamin analysis involve the measurement of a characteristic property of the vitamin or a product formed by it with an analytical reagent. The principal properties which have lent themselves to quantitative measurement are color, spectral absorption, and fluorescence. In the early stages of the developmental history of the various procedures, visual methods of measurement predominated. The coordination of many vitamin assay techniques with suitable instrumentation has brought about improved specificity and precision to the point where, in many determinations, the limit of accuracy is fixed by the errors inherent in the instrument.

The purpose of this section is to provide a brief discussion of the types of measurements upon which the principal physicochemical methods of vitamin analysis are based. A few representative instruments of each type are presented in detail. Neither a thorough theoretical discussion nor a complete coverage of all or most instruments is attempted; it is rather proposed to restrict the discussion to those principles and details which form the general basis of the methods employed in carrying out measurements. Instruments such as the various electrotitrators available for the instrumental determination of titration end points are not discussed here because their application to vitamin analysis is restricted to isolated and special cases. Occasional reference to more comprehensive treatments of the subject will provide a starting point for further study of these problems.

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I. The Measurement of Color

1. Theory

In the most general case, when radiant flux P_1 falls upon an object some of it is reflected, P_2, from the surface, some of it is absorbed, P_3, and the remainder, P_4, is transmitted, with or without scattering.

Radiant reflectance r is the ratio (P_2/P_1) of the reflected radiant flux to the incident flux. Radiant transmittance t is the ratio (P_4/P_1) of the transmitted radiant flux to the incident flux. Radiant absorptance A is the ratio (P_3/P_1) of the absorbed radiant flux to the incident flux. Absorptance and transmittance are of most concern in vitamin analysis. While it is clearly incorrect (*cf.* 1b) to compute absorptance as unity minus the sum of reflectance and transmittance (since this quantity includes losses by internal reflection of light scattered in the medium), or to consider the absorptance of an object observed by transmission as the difference between unity and the transmittance (since this quantity includes the losses by reflection as well as the losses within the object resulting from absorption and scattering), conditions are designed in instrumental analysis to permit the estimation of absorptance by measuring transmittance (*cf.* 2).

The fundamental process that occurs in the emission or absorption of light by an individual atom or molecule is its transition from a state of one definite energy to another state of lower or higher energy. If the molecule, under the influence of radiation, undergoes a change from a state of energy E_1 to a higher state of energy E_2, it will absorb monochromatic light of frequency $\tilde{v}$ such that $E_2 - E_1 = h\tilde{v}$, where h is the universal Planck's constant indicative of the "quantized" character of this energy absorption. It may therefore be seen that the amount of light absorbed by an object or medium under the influence of a constant radiation is proportional to the number of absorbing molecules in the object or medium.

This general fact has been expressed in two quantitative laws. Bouguer pointed out, in 1729, that, of the radiant energy that traverses even a transparent material, a certain fraction is absorbed, and it is reasonable to expect that equal layers of the homogeneous medium will absorb equal fractions of each kind of energy entering them. This statement is known as *Bouguer's* (or sometimes incorrectly (*cf.* 1b) as Lambert's) *law.* If this law is applied to infinitesimal layers and the losses are integrated, the radiant flux at a distance x within the medium is found to be

$$P = P_0 e^{-kx},$$

where P_0 is the flux entering the first layer and k is a constant, known as the absorption coefficient. Most substances absorb selectively, which merely means that k is a function of wavelength λ. This equation can be expressed logarithmically, to the base 10:

$$\log P/P_0 = -Kx$$

or

$$\log P_0/P = Kx$$

where $K = k/2.303$, is usually termed the extinction coefficient.

Bouguer's law is frequently applied to solutions, in which case, either the concentration or the thickness may be varied. Beer drew attention to this rule in 1852, and it is confirmed by observation whenever the process of dilution does not affect the solute or solvent and the latter is either nonabsorbing or a suitable correction is made. If c represents the concentration, *Beer's law* states that, with monochromatic light, the radiant flux transmitted by a layer of thickness x is

$$P = P_0 e^{-kcx},$$

where k is the molar absorption coefficient, a characteristic of the solute, independent of its concentration, but dependent on solvent and temperature. Experimental results are usually reported in terms of the molar extinction coefficient ϵ, so that the above equation, expressed logarithmically to the base 10, becomes:

$$\log P_0/P = \epsilon cx.$$

Hague (2) has pointed out that, in the development of these formulae, two experimental conditions have been imposed, namely, that the incident light be monochromatic in character, and that the incident light be measured through a "blank." Only a few instruments designed to use mercury vapor lamp line spectra strictly obey the first condition. In instruments which isolate wider bands, the attainment of reproducible results depends on a stable combination of illuminant, filter or monochromator, and spectral sensitivity and illumination-current relationship of the measuring cell. Concerning the second condition, the 100% transmittancy point may be set through the blank, provided that it is available at the same time as the sample. It is more common procedure, however, to obtain the 100% transmittancy setting against water or a suitable reference liquid, and to correct the readings obtained against this setting for a blank (both chemical and background) determined on a sample of like type containing none, or a low known concentration, of the substance being determined. For examples of this procedure, see the section on destructive irradiation of vitamin A in the chapter on physical methods of vitamin analysis, and that on niacin assay in the chapter on chemical methods of vitamin assay.

Deviations from Beer's law are frequently encountered over part of the concentration range of an instrument due to solute-solute interactions in more concentrated solutions, association, dissociation, or complex formation or other effects of dilution on the solute or solvent. For this reason, it is common practice to ascertain the range of an instrument over which Beer's law is obeyed and to restrict the use of a calibration constant for calculation to this range. It should be pointed out, however, that a nonlinear calibration curve of density against concentration can be relied upon as well as a linear one, so long as the apparatus remains unchanged (*cf.* 3). In this case, the relationship between instrumental reading and concentration is obtained by reference to the calibration curve rather than a calibration constant.

For an extension of these brief remarks, the reader should consult standard works (1, 3-6), which are also the sources for much of the above material.

2. INSTRUMENTS PRESENTED IN DETAIL

In a general survey of the Committee on Colorimetry of the Optical Society of America (1e), it was pointed out that two classes of different instruments are commonly called colorimeters. Strictly speaking, the term is applicable to instruments employed for the measurement of color, with emphasis on the visual equivalence of the sample to a synthesized stimulus; the physical specifications of the components of the synthesized stimulus provide scales for the psychophysical specification of the visually equivalent color. Mellon (6) terms these instruments "color stimulimeters."

The second class of instruments, called colorimeters by chemists, is employed for the determination of chemical composition or concentration by color comparisons. The term "color comparators" has been recommended for these instruments. Mellon (6) has aptly stated that color absorptometers, which establish the light-absorptive capacity of a system by measuring the light transmitted by the system relative to that incident upon it, have in recent years often been classed as colorimeters along with the color comparators. This subdivision of colorimeters into *color stimulimeters, color comparators,* and *color absorptometers* is both logical and practical and is, therefore, followed here. The instruments which have come to be most widely accepted for analytical work in the vitamin field are the color absorptometers, which may be subdivided into *filter photometers,* which determine the relationship between transmitted light and incident light in a fairly narrow spectral region achieved by means of filters placed between light source and reaction vessel, and *spectrophotometers,* in which these filters are replaced by a monochromator which enables the operator to measure the proportion of transmitted to incident light at any wavelength and with any desired width of spectral band down to the limit of satisfactory operation. Color comparators are also briefly illustrated, since they have enjoyed widespread usage in early methods of analysis and are still of importance to the analyst in some cases.

A. COLOR COMPARATORS

A great variety of devices for color comparison, ranging from Nessler tubes and comparator blocks to more complex instruments such as the Kennicott-Campbell-Hurley colorimeter have been developed and widely applied. The reader is referred to standard texts for a survey of this field (3, 5-8).

The most common instrument of this type is the *Duboscq colorimeter,* which makes use of the principle of balancing the color intensity of an unknown solution against that of a single standard solution. The depth of one solution is kept constant while that of the other is changed until a match in the brightness of the colors is obtained. In systems that conform to Beer's law, the concentrations (C) of the chromogen in the two solutions are inversely proportional to the depths (L) of the solutions, so that $C_1L_1 = C_2L_2$ when the match is obtained.

Figs. 1 and 2 show the actual appearance and diagrammatic view of the instrument.

The following components are indicated in the diagram: A, eyepoint; B, eye lens; C, collective; D, cover glass; E, bi-prism; F, rhomboid prism; G, plungers; H, cups; I, mirror; the left striated column represents the portion of the original beam which reaches the eye, the right striated column that rejected by refraction of the bi-prism. The essential principle of the instrument is as follows: light from an even source of illumination is passed through the two sides of the instrument. There are interposed in these two light paths the solutions which are to be compared. Some of the light, in passing through the liquids, is absorbed, the amount of absorption depending on the depth and concentration of the solution. The two beams of light are then brought to a common axis by means of the rhombohedral prisms. Light from one cup illuminates one-half of a circular field and light from the other cup illuminates the other half. The eyepiece, by which the observer sees both fields with one eye, is focused on the line of separation of the two fields. The depth of one of the two columns of liquid is then altered until the two halves of the field are identical in intensity. When this condition holds, the concentrations of the two solutions are inversely proportional to the depths, which are read on the scales of the instrument.

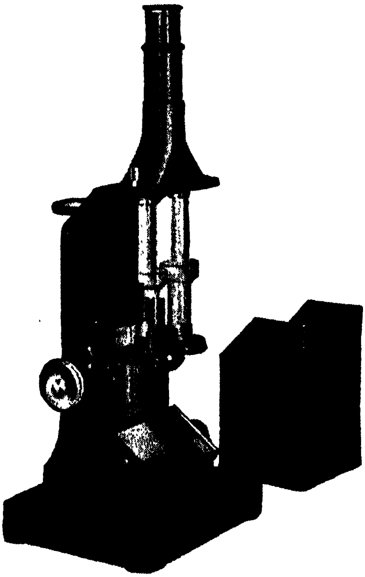

Fig. 1.

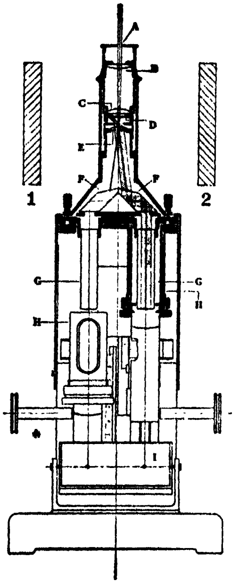

Fig. 2.

Fig. 1. Duboscq colorimeter. (Courtesy Bausch and Lomb Optical Co.)
Fig. 2. Schematic view of same.

Gibb (5) points out the desirability of using an artificial light source rather than daylight to insure uniformity of illumination; the use of a filter to provide as monochromatic light as possible has also been recommended (9, 10). For further details, see the manufacturer's bulletins (11, 12).

B. FILTER PHOTOMETERS

Photometers, rather than compare the relative amounts of light transmitted through two colored solutions, one of known and the other of unknown concentration, measure the amount of transmitted light relative to the incident light without the need for a standard solution. For best results in transmission measurements, the wavelength of the incident light should be the same as the wavelength of maximum absorptance of the solution under investigation. Filter photometers usually provide a wavelength band 30-50 mμ wide, while spectrophotometers are capable of isolating a much narrower band, down to the limit of satisfactory operation.

The earliest and simplest filter photometers were designed for visual measurements. The most common instrument of this type is the *Pulfrich photometer*. Fig. 3 shows the diagrammatic view of this instrument.

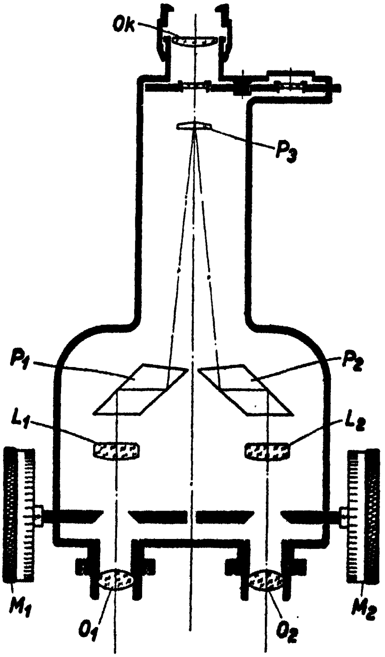

Fig. 3. Schematic view of Pulfrich photometer. (Courtesy Carl Zeiss, Inc.)

The light beams provided by the lamp enter the two cells, O_1 and O_2, one containing the solvent (*i.e.*, permitting 100% transmission), the other containing the solution. The transmitted light beams enter two objectives, L_1 and L_2, with parallel optical axes spaced 2.8 in. apart, then are deflected by prisms P_1 and P_2 into bi-prism P_3. The center rays of these light beams intersect midway of the edge of the bi-prism, and pass through one of a series of color filters mounted in a filter revolver into the eyepiece Ok. The Pulfrich photometer is a so-called diaphragm photometer; *i.e.*, the measurable enfeeblement or reduction of the light passing through the solvent to match that passing through the solution is obtained by varying the aperture of the diaphragms; this is effected by turning the measuring drums M_1 and M_2. By means of the scales on these drums, the diaphragm apertures are related to per cent light transmission through the solution. For further details, reference should be made to the manufacturer's bulletins (13).

The application of photoelectric cells and the general incorporation of selective color filters into instruments of this type brought about considerable refinements in the measurement of the intensity of transmitted light. The fundamental physical principles involved in the application of the photoelectric method to colorimetric measurements have been well summarized by the Evelyn bulletin (14) as follows. When light (rendered approximately monochromatic by passage through a color filter) falls on a self-generating photocell connected in a circuit of low resistance, a current is produced whose magnitude is accurately proportional to the intensity of the light beam (provided this intensity is kept very low). If a galvanometer of very low internal resistance is included in the circuit, the deflection indicated on the instrument is, therefore, directly proportional to the intensity of the light falling on the photocell. The beam of filtered light is directed upon the photocell through a glass absorption cell containing the sample under test. Since the concentration of the desired constituent is proportional to the absorptance of the solution, the galvanometer deflection is a measure of concentration.

Adequate discussions of photocells, as well as of the major structural elements of these instruments are available (1b, 3, 5, 6, 15). The numerous instruments developed in this field may conveniently be divided into one-cell (direct-reading) and two-cell colorimeters. In direct-reading instruments the essential components are a light source, a selective filter of appropriate wavelength band, a tube or vessel to contain the sample, a photocell to receive the transmitted light, and some means of measuring the response of the photocell. The instruments are set for 100% transmission with the solvent or a suitable reference liquid.

This type of instrument is of particular importance in the case of color reactions in which the maximum intensity of color is of short duration, such as the Carr-Price reaction for vitamin A. Provision must be made for constancy in the light source in order to provide a completely reproducible intensity of incident light. Two-cell instruments avoid the necessity of providing constant operating current since fluctuations would affect the two cells equally and thus be compensated. The null-point method of balancing the cells against each other, as indicated by a galvanometer, is also supposed to eliminate errors due to temperature changes or cell fatigue (*cf.* 6).

Two common one-cell colorimeters are the Sheard-Sanford photelometer and the more recent Evelyn photoelectric colorimeter. The *Sheard-Sanford photelometer,* shown in Figs. 4 and 5, is a filter photometer employing a barrier-layer type photocell to measure changes in light transmission between a blank and a solution containing an unknown concentration.

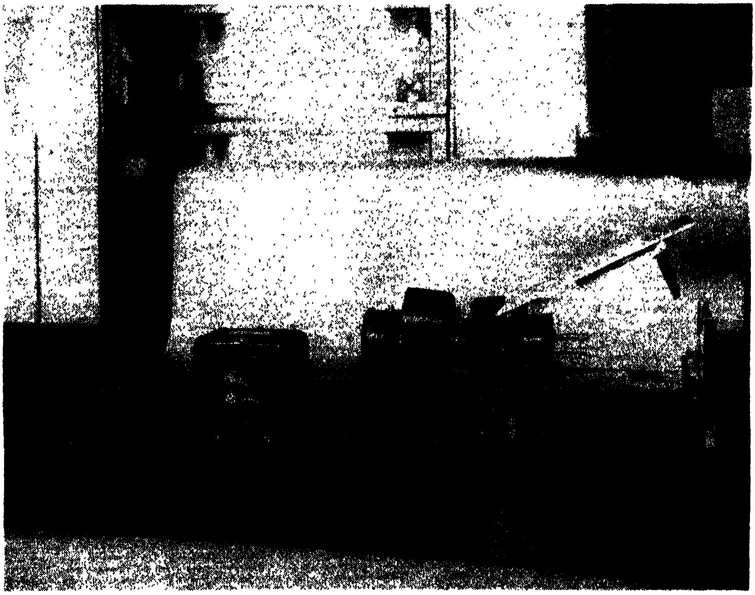

Fig. 4. Cenco-Sheard-Sanford photelometer—side view. (Courtesy Central Scientific Co.)

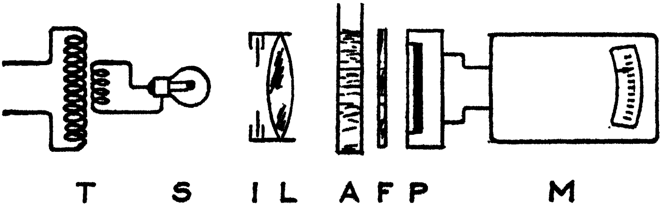

Fig. 5. Schematic view of same.

The main elements of the instrument are (1) a 6-volt incandescent lamp operated by a transformer of constant wattage output to provide light of unvarying intensity; (2) a diaphragm to control the amount of light falling on the photocell, thus permitting a reference setting of 100 regardless of the density of filter employed; (3) a receptacle for holding absorption cells; (4) a suitable filter to isolate a spectral region corresponding to the absorption band of the solution to be analyzed; (5) a barrier-layer type photocell in combination with a sensitive, low-resistance microammeter to indicate the light intensities impinging on the photocell.

The 100 setting is obtained with the solvent in the reference cell.

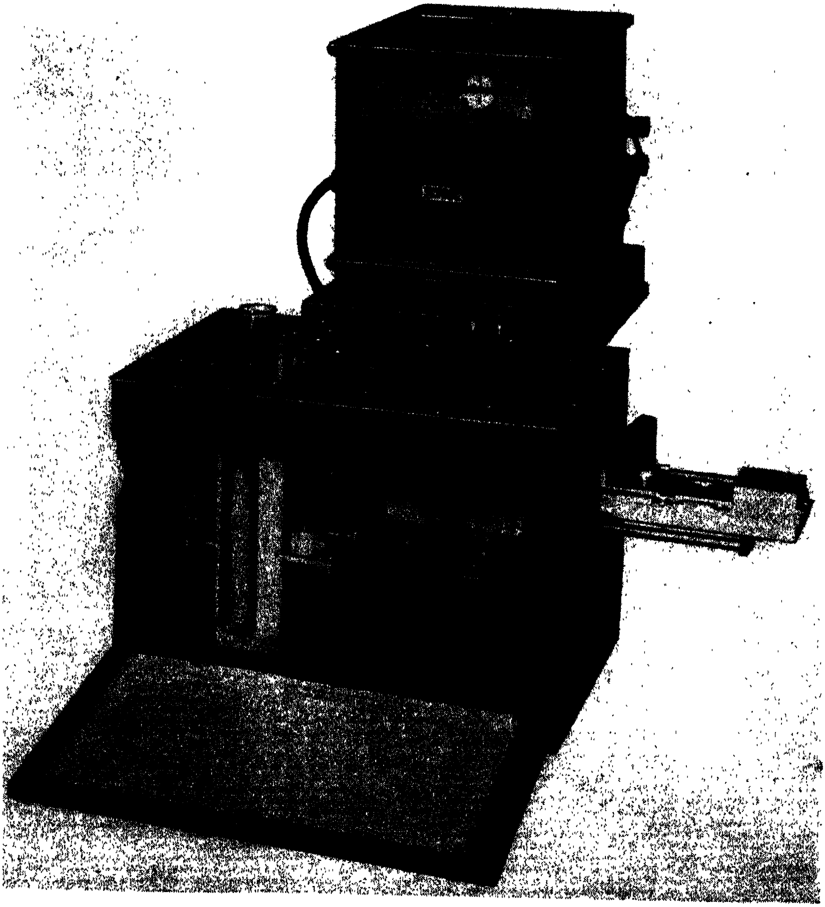

Fig. 6. Evelyn photoelectric colorimeter. (Courtesy Rubicon Co.)

Galvanometer readings and concentrations are related by means of a calibration curve plotted on semi-logarithmic paper. The reproducibility of readings is stated to be 0.2% transmittancy. For further details, reference may be made to the manufacturer's bulletin (16).

A widely used instrument of this type is the *Evelyn photoelectric colorimeter*. Fig. 6 shows the actual appearance of the whole instrument with both the macro and the micro unit in place; Figs. 7 and 8 illustrate the main structural unit.

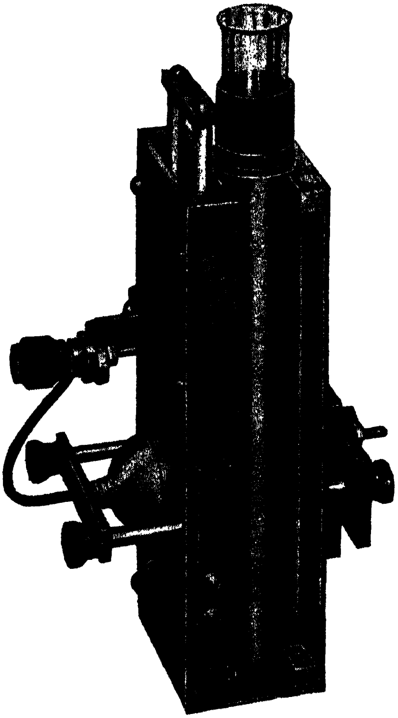

Fig. 7. Main structural unit of Evelyn photoelectric colorimeter.

A beam of light from the lamp L in the reflector R passes through the glass color filter F, and then through the colored solution in the absorption test tube T, before falling on the photocell P. The current from the photocell is registered on the galvanometer shown in Fig. 6. A conversion table is used to convert galvanometer readings (G) to photometric density (L) according to $L = 2 - \log G$, if such conversion is desired. The lamp is energized by a 6-volt storage battery, and the intensity of the light beam is regulated by two rheostats which provide coarse and fine control, respectively. The filters are mounted in filter holders (two filters to each holder) which may be inserted in a slot between the lamp and the absorption tube holder. The test tube fits into a bakelite sleeve S which is pierced by two rectangular apertures A, which define the cross-section of the light beam. The size of these apertures determines the minimum volume of solution on which a measurement can be made.

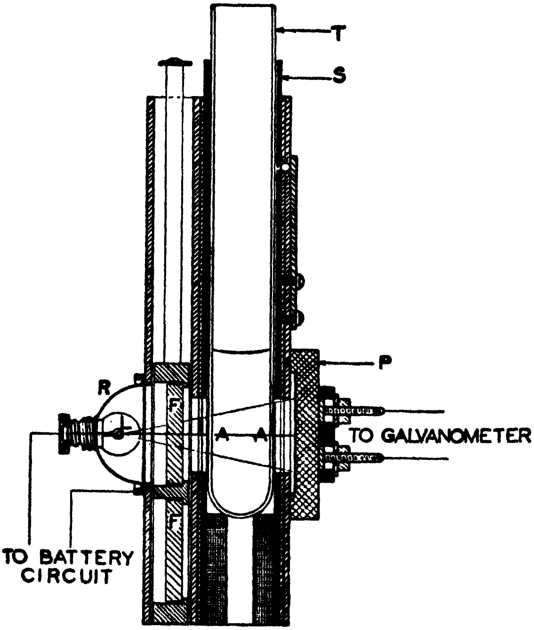

Fig. 8. Schematic view of same.

The source of light consists of a selected Mazda bulb mounted in a hemispherical reflector. The efficient utilization of the luminous flux generated by the lamp permits the use of a bulb in which the power consumption (1 watt) and the current drain (200 milliamperes) are so low that it may be energized by a 6-volt storage battery with its attendant advantage of high stability of light intensity; thermal disturbances encountered with more powerful light sources are also absent.

The use of interchangeable, specially selected round-bottomed test tubes as absorption cells has led to a simplification of the analytical manipulations. In many cases the entire colorimetric reaction is carried out in the tube employed for the final reading, thus eliminating the need for transfers; large series of readings are conveniently run in this way, and the problem of cleaning the reaction vessels is also simplified.

The color filters isolate bands of wavelengths 20 to 60 mμ wide. Filters for any range of the visible spectrum can be secured to fit the requirements of analytical procedures.

A micro unit, essentially analogous to the macro unit, may be incorporated into the instrument to extend the application of the photoelectric technique to procedures in which only small amounts of solution, such as 1 cc. or less, are available (14).

A large number of contributions in the literature have dealt with the application of this instrument to various methods of analysis. Reference may be made to the chapter on chemical methods of vitamin assay for examples of recent procedures successfully based on the Evelyn photoelectric colorimeter. This instrument is also widely used to carry out turbidimetric measurements in microbiological tests, employing filters which do not allow the wavelengths of the color of the solution under test to register. A mathematical and experimental study of the errors inherent in the operation of the instrument (17) indicated that the highest accuracy is obtained when conditions are so arranged that the readings fall on the central portion of the galvanometer scale.

Examples of widely used two-cell instruments are the Aminco Type

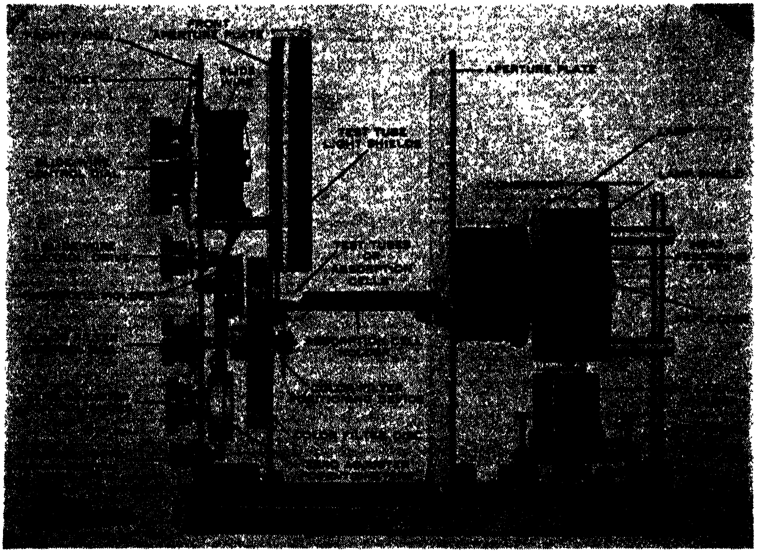

Fig. 9. Interior view of Aminco Type F photometer. (Courtesy American Instrument Co.)

F photometer and the Lumetron and Klett-Summerson photoelectric colorimeters. The *Aminco Type F photometer* is shown in Figs. 9 and 10.

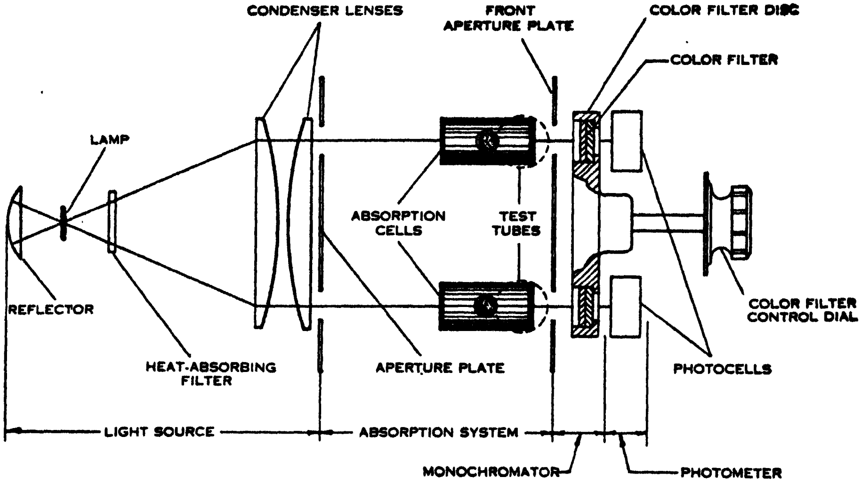

Fig. 10. Schematic view of same.

The photometer in this instrument consists of two self-generating barrier-layer photocells connected in a potentiometric circuit of such nature that the readings obtained are directly in per cent transmission. The light source is an incandescent lamp of the prefocus type. The absorption system employs ordinary selected 7 × ⅞ in. test tubes or 16 mm.o.d. precision absorption cells as solution holders; cells as short as 1 mm., and having a volume of approximately 0.14 ml. are available for micro measurements. A series of six filters is mounted in a disc in such a manner that the operator need merely turn a knob to the required position to change from one filter to another.

The following procedure is used in making a measurement. An absorption cell or test tube containing the solvent is placed in each beam. The large dial at the top of the panel is set to read 100, and the coarse and fine adjusting knobs turned until the galvanometer indicates zero current. A third cell containing the colored solution to be measured is then substituted for the right-hand cell. If light is absorbed, the galvanometer will deflect. The large dial is then turned until the galvanometer again indicates zero. The dial position under these conditions indicates the per cent transmittancy of the solution at the wavelength used. The use of two photocells enables the instrument to compensate automatically for ordinary line-voltage changes of about 3%. For further details, reference may be made to the manufacturer's bulletin (18).

The *Lumetron photoelectric colorimeter* is shown in Fig. 11.

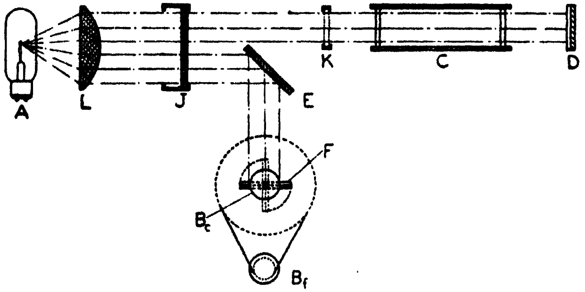

Fig. 11. Schematic view of optical system of Lumetron photoelectric colorimeter, Model 402-E. (Courtesy Photovolt Corp.)

Light from the 100 cp projection lamp A is collimated by the optical system L to form a parallel beam which is split into two parts. One part of the beam passes through the sample holder C and strikes the measuring photocell D. The other part of the beam is deflected by the mirror E and acts upon the balance photocell F which is mounted so that it can be turned through an angle of 90°. In the end position, the cell is normal to the beam and receives its full light, in the other end position, it is parallel to the light beam so that there is no action of the light upon the cell. The position of the cell can be adjusted by the knobs B_c (coarse) and B_f (fine).

The removable filter holder J is designed to accommodate any combination of 2″ square filters up to 1″ combined thickness. The filter serves both photocells because the light passes through it before being split into the two beams. A clamp K is provided for an additional filter to be traversed only by the light acting upon the measuring cell. If a neutral gray filter is inserted into this clamp, this filter can serve as a multiplier.

The self-generating barrier-layer photocells are connected in a bridge circuit, the balance of which is indicated by the galvanometer. The circuit represents a current bridge rather than a voltage bridge. The zero indicator of the bridge circuit is a taut-wire spot-light galvanometer which has a low coil resistance of 90 ohms and a high sensitivity of 0.06 microampere per division. Electrical balancing of the bridge is achieved by turning the adjustable contact arm of a slide-wire which is provided with coarse and fine adjustment knobs. The filters isolate wave-bands approximately 30 mμ wide. Further details are given in the manufacturer's bulletins (19).

The Klett-Summerson photoelectric colorimeter is shown in Figs. 12 and 13.

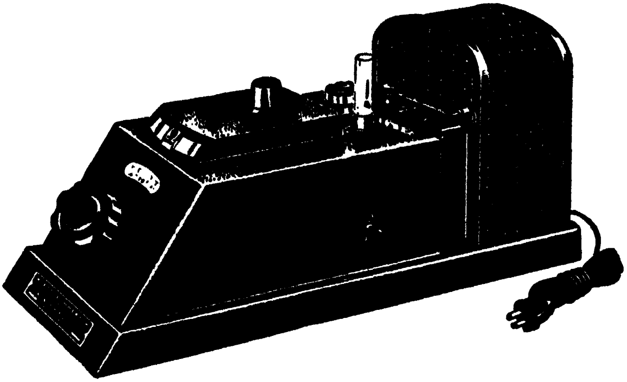

Fig. 12. Klett-Summerson photoelectric colorimeter. (Courtesy Klett Mfg. Co.)

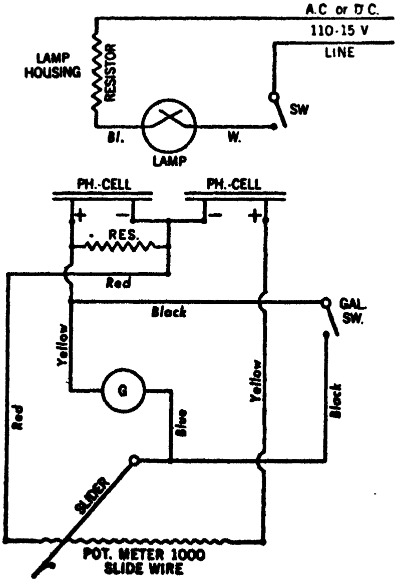

Fig. 13. Schematic view of same.

Light from a 100 watt projection lamp activates each of two "blocking layer" photoelectric cells mounted at the rear of individual enclosed compartments, the light entering the compartments through rectangular slits in the face of each compartment opposite the photoelectric cell. Through an opening in the top of one compartment the test tube containing the solution under examination is interposed in the path of light striking one of the two photoelectric cells, called the working cell.

The second photoelectric cell, called the reference cell, is continuously activated by the source of light and is in no way affected by the procedure of measurement on an unknown solution. The sole purpose of this cell is to furnish a source of current against which changes in the current output of the working cell may be measured. It is the equivalent of the standard cell in the usual potentiometric circuit, whereas the working cell corresponds to the "unknown" cell.

Color measurements are in terms of the scale attached to the potentiometer. The scale records the position of the sliding contact with respect to the full length of the potentiometer resistance. Measurements are carried out by placing the reference fluid (solvent or reagent blank) in the instrument, setting the potentiometer scale to read zero, and bringing the two photocells to current equivalence through the galvanometer. When the reference fluid is replaced by the fluid under examination, any decrease in current output of the working cell caused by a difference in light transmission between the two fluids is established by varying the position of the sliding contact on the potentiometer until potentiometric balance has been restored. The reading on the potentiometric scale at this point is the measure of the color intensity of the fluid under examination. The instrument may also be used as a "turbidimeter" for microbiological assays with the appropriate filters.

Test tubes or glass cuvettes may be used as reaction vessels. Selective light filters, of 50-75 mμ width, are used routinely with this instrument. The scale of the galvanometer is so constructed that scale readings are directly proportional to the concentration when Beer's law is obeyed. Further details are given by Summerson (20) and in the manufacturer's bulletins (21).

C. SPECTROPHOTOMETERS

The essential difference between filter photometers and spectrophotometers is that the latter employ a monochromator instead of a color filter for the isolation of the desired wavelength from the light source. While filter photometers provide only an approximation of monochromatic light, since the narrowest band they can isolate is about 20 mμ wide, spectrophotometers enable the investigator to use truly monochromatic light and to determine, wavelength by wavelength, the proportion of transmitted to incident light.

Spectrophotometers, like filter photometers, have been developed along various lines. Visual instruments, such as the König-Martens, the Hilger-Nutting, the Gaertner, and the Bausch and Lomb spectrophotometers (*cf.* 6), have been widely used, but have obvious disadvantages when compared to photoelectric instruments. One-cell and two-cell instruments are available, and the advantages and disadvantages adduced for both are analogous to those mentioned for filter photometers. Most spectrophotometers are of the non-recording, *i.e.,* manually operated type, but a common example of the recording type is available in the instrument developed by General Electric and described below. Examples of typical applications of these instruments to vitamin analysis are given in the chapter on physical methods of vitamin analysis.

A widely used photoelectric spectrophotometer is the instrument designed by Cary and Beckman (22). Figs. 14 and 15 show the *Beckman Model DU Spectrophotometer.*

An image of the light source A is focused by the condensing mirror B and diagonal mirror C on the entrance slit at D. The entrance slit is the lower of two slits placed vertically over each other. Light falling on the collimating mirror E is rendered parallel and reflected toward the quartz prism F. The back surface of the prism is aluminized, so that light refracted at the first surface is reflected back through the prism, undergoing further refraction as it emerges from the prism. The collimating mirror focuses the spectrum in the plane of the slits D and light of the wavelength for which the prism is set passes out of the monochromator through the exit (upper) slit, through the absorption cell G to the phototube H.

The monochromator is of the autocollimating type with 30° prism of selected crystal quartz, and 50 mm. effective diameter. The back surface is aluminized. The aluminized collimating mirror is 50 × 90 mm., with a 100 cm. radius of curvature. The prism mount is supported on kinematic bearings and rotated by an arm engaging in a four-turn precision spiral. The wavelength scale is 1 m. long, calibrated 200 to 2000 mμ and easily readable to 0.1 mμ in the ultraviolet and 1.0 mμ in the infrared.

The curved bilateral entrance and exit slits of 13 mm. effective height are simultaneously and continuously adjustable in width from 0.01 to 2.0 mm. by parallelogram-cam mechanism. Adjustment can be made within 0.1%, even at narrow slit widths. The calibrated dial is direct reading in millimeters.

Interchangeable light sources are mounted in quick-change housings. For measurements down to 320 mμ a 6 volt, 25 watt tungsten filament lamp is used, operated from a storage battery. For measurements in the ultraviolet region below 320 mμ a hydrogen discharge lamp is used, oper-

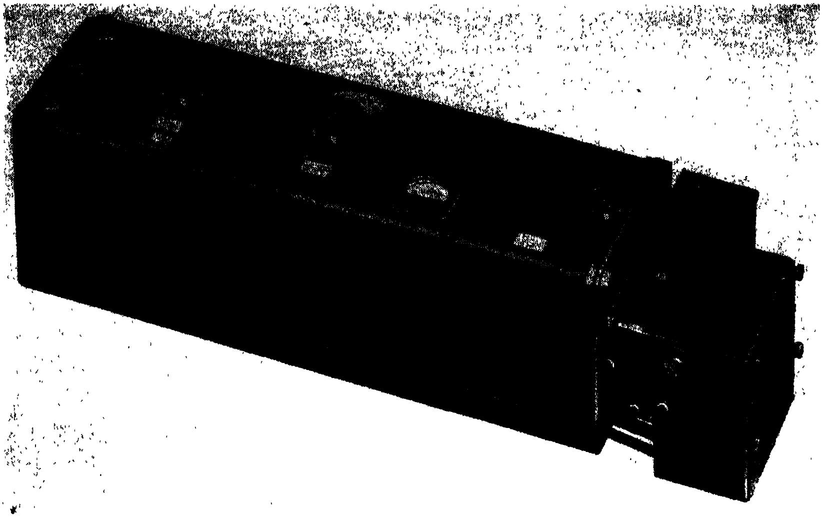

Fig. 14. Beckman Model DU spectrophotometer. (Courtesy National Technical Labs.)

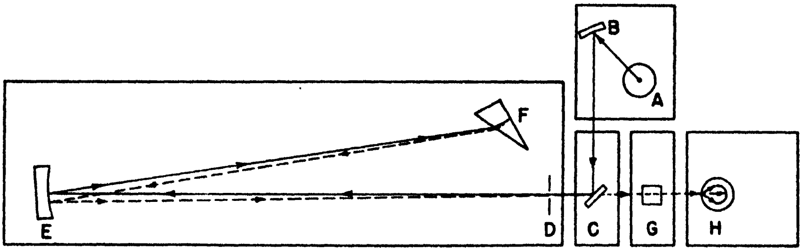

Fig. 15. Schematic view of optical system of same.

ated by a 110 volt, 50/60 cycle power supply unit with electronic voltage control which stabilizes light output within 0.2%, regardless of normal line voltage fluctuations.

A cesium oxide phototube with high sensitivity from 1000 to 600 mμ and a blue-ultraviolet-sensitive phototube in an ultraviolet-transmitting envelope, with high sensitivity from 650 to 200 mμ are supplied with the instrument. A sliding rod brings either tube into position and simultaneously switches their electrical connections.

The phototube current is measured by balancing the voltage drop across a 2000 megohm load resistor with a slide wire potentiometer. An electronic amplifier having a current sensitivity of 2×10^{-13} amperes indicates circuit balance on a milliammeter. The potentiometer scale is calibrated in both per cent transmission and optical density.

The adaptation of the Beckman spectrophotometer to measurements on minute quantities of biological materials has been reported (23). A recent critical evaluation of the instrument includes the description of a constant-temperature enclosure for the samples (24). For further details, reference should be made to the manufacturer's bulletins (25).

A number of photoelectric spectrophotometers have been developed by Coleman. An older model, providing a double monochromator for isolation of narrow band widths, is no longer in production; the Junior Clinical Spectrophotometer operates on the same principle as the model to be described here.

The Coleman Universal Spectrophotometer (Model 11) is shown in Fig. 16.

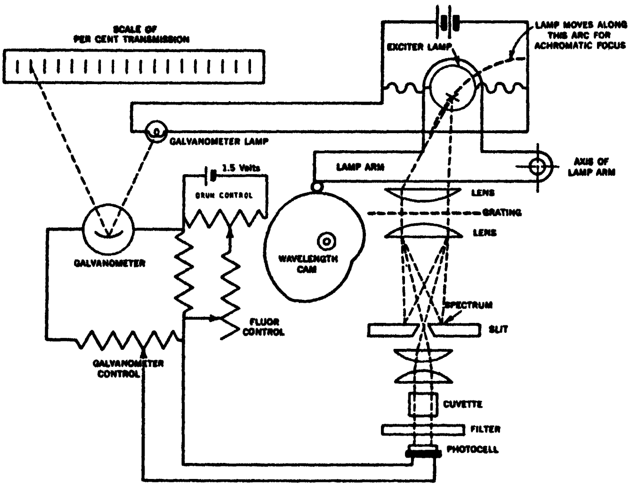

Fig. 16. Schematic view of Coleman Model 11 Universal spectrophotometer. (Courtesy Coleman Instruments, Inc.)

Transmittance may be measured by this instrument either by direct reading from the galvanometer scale or, more accurately, by a null method, reading directly from the drum scale. The optical system consists of a 32 watt, 8.5 volt concentrated filament lamp, an ultraviolet transmitting lens system, Wood's Echelette Diffraction Grating and a fixed exit slit. A 35 mμ band may be selected anywhere within the region of 300-800 mμ. The instrument is operated with a constant-voltage transformer from any suitable 110-125 v. 60 cycle line, or preferably from an 8-volt storage battery.

The Universal model accepts two different cuvette carriers, both of which are designed to take several sizes of cuvettes. One carrier will accommodate round cuvettes, 19 mm. test tubes and the square 13.08 mm. fused Pyrex cuvettes, which further accept square prisms whereby the fluid depth may be reduced to about 2 mm. It is introduced through the oblong opening in the instrument panel and may be shifted to locate either reference or sample cuvette in the light beam. The other carrier, which is introduced through the side door of the instrument and slides back and forth on a stainless steel rack, accommodates 25 mm. and 50

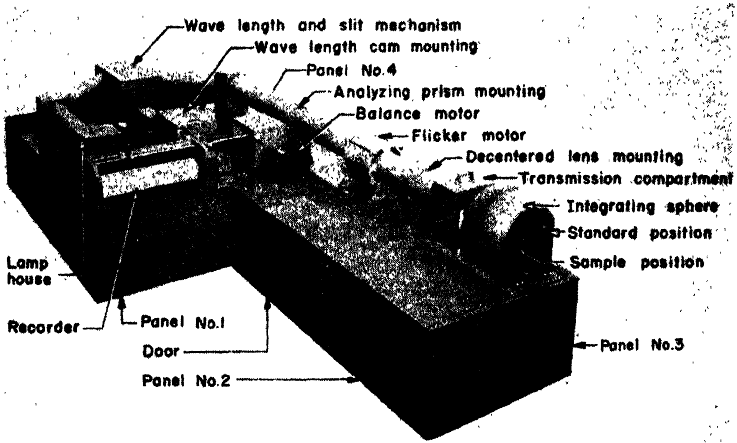

Fig. 17. Hardy-General Electric recording spectrophotometer. (Courtesy General Electric Co.)

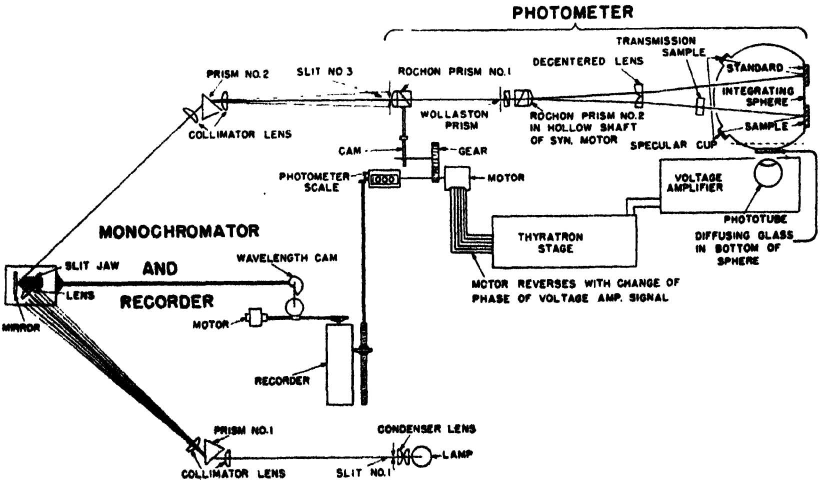

Fig. 18. Schematic view of same.

mm. round fused cuvettes and a 20 × 40 mm. fused oblong cuvette. These longer cuvettes are necessary for the examination of very pale solutions. Further details on this instrument are given in the manufacturer's bulletins (26).

The *Hardy-General Electric recording spectrophotometer* is shown in Figs. 17 and 18.

The spectrophotometer consists of a light source which radiates all wavelengths of the visible spectrum (400-700 mμ), a monochromator for selecting monochromatic light as the spectrum is scanned, a flicker system for shining the light alternately on the sample and on a standard, a phototube and associated amplifier circuits, and a recorder for making a pen-and-ink record of the measurement.

The optical system and recorder are mounted on a flat steel plate with the power supply, phototube amplifier, and thyratron stages directly below. The size and shape of the spectrophotometer make it suitable for table mounting.

The light source (Type C8T10) is a standard screw-base projection lamp which operates from 6 volts, drawing 18 amps. The monochromator is a double-prism type which passes a constant wavelength band of 10 mμ throughout the entire visible spectrum from 400 to 700 mμ. The flicker system consists of three polarizing prisms. One of them, a Rochon prism, is so mounted inside the hollow shaft of an 1800 r.p.m. synchronous motor that the light alternately strikes the standard and the sample 60 times per second. The integrating sphere contains the holders for the sample and the standard.

The phototube used in the spectrophotometer is the Type PJ14B. The instrument operates from 115 volts, 60 cycles A.C., and requires about 400 watts. The recorder is a revolving drum on which the wavelength scale (around the drum) is 6⅞ inches long, and the transmission (or reflectance) scale, along which the pen moves, is 10 inches long. The pen is driven by the balance motor, while the drum is driven by the wavelength motor. The normal time for recording a curve is 2.5 minutes. A low speed, which gives a curve in 5 minutes, is also provided for steep curves. The photometer cams govern the movement of the pen. With one cam, full scale represents 100% transmission (or reflectance) and with the other cam, full scalc represents 20% transmission (or reflectance). Other cams are also available.

The method of measurement is shown in Fig. 18. The light from the projector lamp is dispersed by prism No. 1. The slit and mirror select a band of light 10 mμ wide. The mirror and slit are so driven by the wavelength motor through a cam system that the spectrum is continuously scanned, from 400 to 700 mμ. Another cam system so varies the mechanical width of the slit that the 10 mμ band can be kept constant through the spectrum. This band of light is further dispersed by prism No. 2 and trimmed by slit 3 to insure its spectral purity.

The monochromatic beam is then passed through the polarizing and flickering optical system into the integrating sphere where it falls alternately 60 times a second on the sample and the standard. If the light reflected by the two is equal, the phototube sees a constant light level. If, however, the reflectance is not equal, the phototube will see light variation at the rate of 60 cycles a second.

This 60-cycle voltage signal from the phototube is amplified and applied to a thyratron stage, to drive a small split-field D.C. motor. The motor is connected through the photometer cam to the Rochon prism No. 1. This "balance" motor rotates the prism, which changes the light intensity on the standard with respect to the sample, until the light reflected by the two is equal, at which time the phototube sees no light variation and stops the motor.

The position of the Rochon prism is then a measure of the transmission of the sample compared with that of the standard. The recording pen is driven by the same shaft that drives the prism; therefore, the position of the pen is an accurate measure of the difference in transmittance between the standard and the sample. The measurement is always made at the point of the zero voltage signal and is, therefore, independent of the characteristics of the phototube, lamp, or amplifier. Further details are given in the manufacturer's bulletin (27).

II. The Measurement of Fluorescence

1. THEORY

When a beam of light passes through a medium, light energy may be absorbed by molecules in the medium. Many substances can reëmit light following such an absorption. This phenomenon is called luminescence. If the emission of light does not continue for any measurable time after the source of excitation has been removed, the phenomenon is known as fluorescence; if the emission of light continues beyond the period of excitation, the term phosphorescence is used.

According to the quantum theory, electromagnetic radiations such as light are streams consisting of discrete packets or quanta of energy possessing a definite wave-characteristic. Molecules which absorb a quantum of light suffer displacement of electrons from their normal orbits to others, probably farther removed from the nucleus. An unstable condition is thus set up, and if the electron returns to its original position, there is an emission of energy. Fluorescence is due to this spontaneous transition of a molecule from an excited state to a lower energy level.

Stokes' law states that the energy emitted as light in this process is always less than the energy initially absorbed, *i.e.*, the emitted wavelengths, corresponding to quanta of lesser energy, are always longer than the absorbed wavelengths which correspond to quanta of greater energy. On the basis of this principle, ultraviolet light has been recognized to possess the characteristics most suitable for analysis by fluorescence. Those compounds for which this type of analysis has been found most practical absorb ultraviolet light and emit light of sufficiently longer wavelength to become visible. However, compounds which absorb visible light and emit visible light of a longer wavelength can also be determined in this way.

It may easily be seen that the amount of light emitted by a solution placed in a beam of incident light depends on the number of molecules capable of absorption and subsequent emission of light energy. The specific fluorescence depends on 1) the concentration of the fluorescing substance or substances, 2) the intensity of the source of excitation, 3) the wavelength of excitation, 4) pH, 5) temperature, 6) the nature of the solvent, and 7) the presence of foreign bodies. The instruments used to measure fluorescence and the analytical procedures are so designed that, ideally, all variables except the concentration of the fluorescing substance are controlled, so that a direct proportionality between concentration and fluorescence is readily available.

Briefly, the general procedure in fluorescent analysis consists of placing the sample in a suitable solvent and after appropriate preliminary treatment, in a beam of light made as monochromatic as possible by light filters, and measuring, either visually or photoelectrically, the fluorescent light which is emitted. The relation between fluorescence and concentration is established by preparing a calibration curve; a standard sample may be run along with each batch of unknowns to correct for variations in the procedure.

For a more extensive discussion of these and related considerations, reference should be made to the works of Radley and Grant (28), Hirschlaff (29), Pringsheim and Vogel (30), and De Menth (31), and to papers by Mueller (32), De Menth (33), Ellinger and Holden (34), and Bowen (35, 36), among others.

2. INSTRUMENTS PRESENTED IN DETAIL

The essential features of the instruments designed to measure fluorescence are 1) a light source providing the type of excitation needed to cause visible fluorescence; 2) a set of light filters for the incident as well as fluorescent light transmitting the needed portion of the spectrum for the system under investigation; 3) a cuvette or tube to contain the test solution; 4) a device to measure the amount of emitted fluorescence: either a direct-reading galvanometer connected to a single photocell or a galvanometer used as a null-point indicator in a potentiometric system utilizing two photocells. Radley and Grant (28) and Pringsheim and Vogel (30) treat these elements separately and in some detail.

Early measurements of fluorescence intensity were made in test tubes illuminated laterally by a source of ultraviolet light. A more advanced type of visual measurement was made possible by the adaptation of nephelometers such as the Duboscq instrument and filter photometers such as the Pulfrich and Leifo models to fluorescence measurements. To use these instruments in this fashion, a strong source of ultraviolet, from which visible light was removed by a filter, was substituted for the usual source of white light (*cf.* 5, 32) and the intensity of the light transmitted by the fluorescing solution was measured.

With the advent of photoelectric photometers, two general types of instruments became available for the measurement of fluorescence. One type employed the above principle of adapting a transmittance instrument to fluorescence measurements by substituting a suitable ultraviolet light source for that used in colorimetry. Illustrative of this type of instrument is the Coleman Universal Spectrophotometer, which can be conveniently adapted to fluorescence measurements by the application of the UV Illuminator.

The second type of instrument was designed exclusively or primarily for the measurement of fluorescence. In these instruments the photocell is placed at the side of the vessel and parallel to the beam of light rather than in the path of the beam of light behind the vessel to minimize the amount of radiation falling on it from the primary ultraviolet beam. In some models, a second photocell is placed in the path of the beam of light behind the vessel to permit the use of the photofluorometer for colorimetric or turbidimetric transmittance measurements. Most structural elements of the instruments in this group are essentially the same; the light source is usually a mercury arc; the type of primary filters (*i.e.*, between light source and vessel) and secondary filters (*i.e.*, between vessel and photocell) differ little from instrument to instrument; the cuvette containing the fluorescing solution, though often different in form and size, is provided for in the instrument in the same way. In the simplest circuits, a barrier-layer photocell is directly connected to a sensitive galvanometer, and the galvanometer deflection is calibrated in terms of solution concentration. Since the fluorescence of sample and standard cannot be measured simultaneously, the light source must be very stable, so that exactly the same intensity of incident light is provided for successive readings. Examples of this type of instrument are the photoelectric fluorophotometers designed by Cohen and Hand (*cf.* 5) and the Pfaltz and Bauer Fluorophotometer described in detail below.

Barrier-layer photocells may also be used in circuits in which the potential developed by the photocell is balanced against an opposing potential from a battery (*e.g.*, Coleman Universal with UV Illuminator) or from a comparison photocell which intercepts part of the incident light beam. This set-up is especially advantageous where provision for a very stable light source cannot be made. Examples of the latter arrangement are the Klett Photoelectric Fluorimeter (37) and the Lumetron Photoelectric Fluorescence Meter described below.

Another type of instrument employs a photoemissive cell in place of the photocell. The current is then amplified by a single-stage electronic amplifier and measured in terms of amplifier plate-circuit current. Sensitivities several times as high as those obtainable with the simpler cell-galvanometric arrangement are claimed and may be expected on the basis of current practice in electronics (*cf.* 32). The Coleman Electronic Photofluorometer is an instrument of this type.

Loofbourow and Harris (38) some years ago discussed and evaluated a number of these instruments with special reference to their applicability to the thiochrome assay for thiamine, one of the most common fluorometric techniques. They concluded that all instruments tested have sufficient sensitivity, linearity, and reproducibility of readings to permit evaluation to within 1.5-2.0%; they also found that the variation in readings due to line voltage fluctuations is of the order of 2% per volt at 110 volts in instruments without balanced photocell circuits; this variation is reduced to the order of 0.1% per volt in instruments employing balanced circuits, or in unbalanced instruments when used with line voltage regulating transformers on lines with good frequency regulation.

The *Pfaltz and Bauer Fluorophotometer, Model B (cf. 39)*, when used for fluorometric methods, operates upon the general principle outlined above. The exciting light source is an 85 watt mercury capillary arc (Type H 3) enclosed in a protective glass bulb. The necessary voltage is supplied by a transformer connected to a 110-120 volt A.C. 60 cycle line. A voltage regulator may be connected between the line voltage and the transformer. The socket which holds the lamp is adjustable vertically while the carriage to which the socket is held is adjustable horizontally.

To the rear of the lamp is a reflector, and in front a condensing lens system. An iris diaphragm, with scale and indicator on the lever, permits control of the light intensity. A holder to accommodate spectral filters is directly in front of the iris diaphragm.

The lamp housing is mounted on a base and is adjustable from side to side by means of a slot and clamping screw to permit accurate alignment of the light beam to the optical axis.

The metal tube is provided with a slot which accommodates filter holders for single filters or filter combinations. This tube is removable from the cuvette housing in order to accommodate other accessories, such as lamp housings or monochromators. The exciting light passes through this metal tube to the cuvette housing, one side of which can be removed for the purpose of observing the light beam passing through the solution. The rectangular form of the cuvette minimizes scattering of the activating light and permits the use of square spectral filters which allow high percentage transmission of the fluorescent light. At either end of the cuvette holder slots are provided to accommodate masks to control the size of the light beam. At the sides are slots to accommodate spectral filters, which are chosen to allow passage of the fluorescent, but not the exciting light.

The distance between the lamp and the cuvette housing may be varied by means of the dovetail slider to which the latter is fitted.

One photocell is exposed to the fluorescent light and is connected with the galvanometer when the switch is in position FL (fluorescence). The other photocell is exposed to the transmitted light and is connected with the galvanometer when the switch is in position TR (transmission). Provision is made for the use of a shunt box in transmittance measurements. For the purpose of calibrating the light intensity the aperture of the iris diaphragm may be adjusted to a definite opening. This light intensity is then read on the galvanometer. It is possible to obtain any desired deflection up to the maximum per unit of the material being measured. Thus, a graph may be made showing the relationship between the transmitted and fluorescent light intensities as measured by the two photocells.

Solutions of quinine sulfate and fluorescein have been used as secondary standards in the determination of vitamins B_1 and B_2. Recently, the use of fluorescent glass blocks for this purpose has been widely accepted. Glass standards of this type are available for thiamine and riboflavin assays.

Figs. 19 and 20 show the components of the *Lumetron Photo-Electric Fluorescence Meter Model 402-EF (cf. 40).*

The light of the mercury vapor lamp is condensed by an optical system to form a parallel beam. This beam passes through a narrow-band filter which isolates the exciting light of the proper wavelength. The exciting beam is then split into two parts. One part enters the sample holder which is provided with a thin front window of low ultraviolet absorption. The fluorescence of the liquid is registered by two large

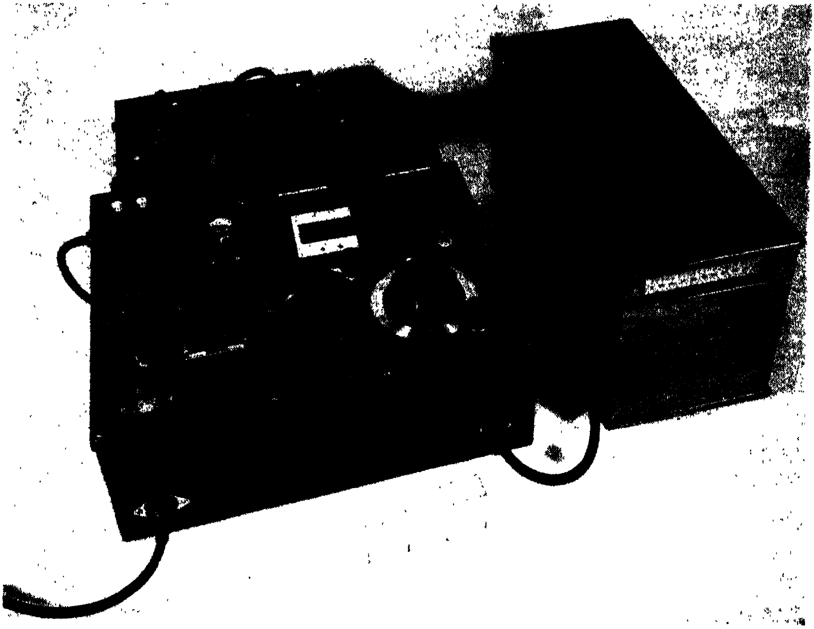

Fig. 19. Lumetron fluorescence meter. (Courtesy Photovolt Corp.)

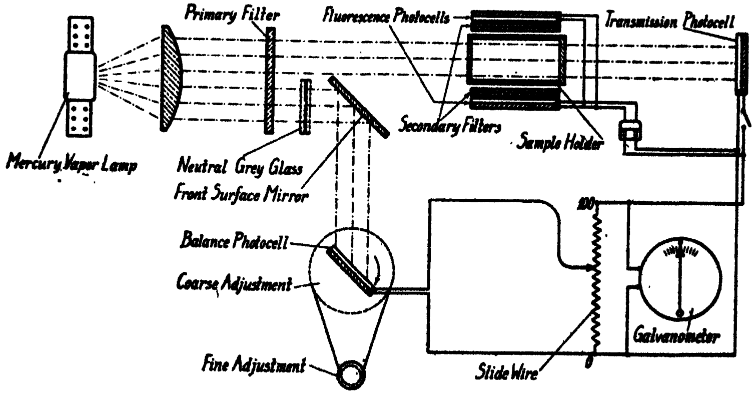

Fig. 20. Schematic view of same.

barrier-layer photocells which are arranged laterally on both sides of the sample holder in the fluorescence pick-up unit. Filters between sample holder and photocells serve to isolate the specific fluorescence band and to eliminate the influence of primary light which may be scattered by particles suspended in the liquid.

The other part of the beam is deflected by a front surface mirror and acts upon the balance cell, which is mounted so that it can be turned through an angle of 90°. The two measuring photocells and the balance photocell are connected in a bridge circuit with slide wire and with a galvanometer as the zero indicator. This bridge circuit eliminates the influence of light intensity variations of the mercury vapor lamp.

This model, in addition to its application for measuring fluorescence, is suitable for colorimetric and turbidimetric tests by transmission in the visible range by substituting an incandescent lamp for the mercury vapor lamp. For use of the instrument for colorimetric tests, a photocell which is provided for transmission measurements is connected into the circuit. Transmission tests can be carried out with sample holders of every description, from microcells to absorption cells of 150 mm. light path. Being equipped with quartz optical system and with a mercury lamp having an envelope of high ultraviolet transmission, the instrument is, furthermore, suitable for ultraviolet transmission tests. Turbidity measurements can be carried out by registering the light which is scattered sideways by suspended particles (nephelometry) or by transmission readings with long sample holders. For reflection tests, the fluorescence pick-up unit is replaced by a reflection adapter.

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